X. THE CONDENSATION OF TRYPTOPHANE AND OTHER INDOLE DERIVATIVES WITH CERTAIN ALDEHYDES.

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(*Received November 26th, 1912.*)

The oxidation of tryptophane by methods other than that adopted by Hopkins and Cole [1902] gives rise to coloured amorphous compounds. These observers, employing ferric chloride as the oxidising agent, isolated two substances, the one having the formula C_9H_7ON and afterwards identified by Ellinger [1906] as β-indolealdehyde; the other an interesting crystalline base, the analysis of which gave results in accordance with the formula $C_{12}H_{10}N_2$. This method of oxidation was used in the course of the present investigation with a view to the isolation and identification of the base $C_{12}H_{10}N_2$. Unfortunately, however, even though the conditions were varied as much as possible, none of this substance could be obtained. Ellinger, when preparing β-indolealdehyde by this method, had also failed to obtain Hopkins' base. In trying to ascertain the extent to which the ether used for extraction of indolealdehyde had been directly or indirectly responsible for the production of the base, Hopkins noticed that a crystalline derivative was formed when an aqueous solution of tryptophane was in contact with ether which had been heated locally with a glass rod. This compound however did not prove to be the much sought after base $C_{12}H_{10}N_2$, but a new substance of an acidic nature. At his suggestion the properties of the new substance, which may be conveniently spoken of as the "ether oxidation substance," were examined. This work led to an investigation of the direct action of formaldehyde, glyoxylic acid and glyoxal on tryptophane, and the action of trioxymethylene, in the presence of a condensing agent, on indole, skatole, indolepropionic acid, indoleacetic acid and tryptophane. It was found that the direct action of formaldehyde and glyoxylic acid on tryptophane led to the formation of *colourless* crystalline compounds; in these cases the *amino*-group of tryptophane was the reactive group (Section A). Glyoxal reacted directly with

tryptophane in the presence of an oxidising agent to form a coloured derivative (Section B). For interaction with the *imino*-group of indole derivatives, the presence of a condensing agent is necessary, and the resulting products are all deeply *coloured* substances (Section C).

Section A.

I. *Preparation of the ether oxidation substance.*

(*a*) An aqueous solution of tryptophane was poured into a large wide-mouthed bottle fitted with a bung, and sufficient ether was added to form a layer a few mm. deep. In preliminary experiments the ether was heated locally by plunging a red-hot glass rod into the bottle and keeping it there a few minutes. Very pungent fumes were evolved, accompanied by a considerable rise in temperature. The process was repeated several times, and the bottle was well shaken and allowed to stand for a short time, when a crystalline deposit slowly formed. This somewhat tedious process was replaced by the following simple arrangement.

(*b*) The bung was pierced with four holes through which were passed a straight wide tube open at both ends, two electrodes, and an exit tube of narrow bore. The electrodes were connected inside the bottle by means of a platinum spiral placed just above the surface of the ether. The wide tube was arranged so as to have one end close to the platinum spiral. One end of the narrow tube was flush with the bung inside the bottle, the other end was connected with the vacuum pump and a slow current of air was drawn through the apparatus entering by the wide tube.

Outside the bottle the electrodes were connected with a battery sufficiently powerful to make the platinum spiral red-hot. The electric current was then switched off, and the current of air was regulated so that the heat developed during the incomplete combustion of the ether was sufficient to keep the platinum wire glowing. As soon as the layer of ether had disappeared, a further supply of ether was added, and the process repeated. The bottle was then disconnected, corked and left to stand at a temperature of 38° for several hours, during which time a crystalline deposit slowly formed. This substance proved to be the same as that prepared in (*a*).

(*c*) In another experiment a mixture of ether vapour and steam was passed over red-hot glass, and the ensuing vapours were passed through a solution of tryptophane; under these conditions no ether oxidation substance

was formed. Thus it is evident that, to bring about the production of this substance, it is necessary for the tryptophane to be in contact with the reactive principle at the moment of its formation. The pungent fumes evolved during the slow incomplete combustion of ether were passed through a wash bottle containing water, and on examination were found to contain both hydrogen peroxide and formaldehyde.

(*d*) An attempt was made to prepare the substance by the action of formaldehyde and hydrogen peroxide on tryptophane. A crystalline derivative was obtained, but it did not prove to be the ether oxidation substance (*post*, p. 107).

In (*a*) and (*b*) the yield of crystalline product was 65 per cent. of the original tryptophane. The substance melts and decomposes at 324°, is colourless, crystalline, slightly soluble in water, alcohol and phenol, insoluble in cold and hot benzene, xylene, toluene, ethyl acetate, aniline, nitrobenzene, pyridine, amyl alcohol and ethylene dibromide. It is soluble in boiling glacial acetic acid: with concentrated hydrochloric acid it darkens considerably, and on evaporation *in vacuo* crystals separate out, which can be recrystallised from ether and alcohol and melt at 285°. Several analyses of this product were made, but failed to give concordant results. The substance can be boiled with dilute hydrochloric and sulphuric acids (5%) without any decomposition taking place. It is precipitated by the mercury sulphate reagent, gives the glyoxylic but not the bromine tests, and does not darken with concentrated sulphuric acid. It is soluble in alkalis, and can be precipitated by means of acetic acid. The use of caustic alkalis is to be avoided, owing to the pigmentation which ensues. Its sodium salt cannot be recrystallised from water owing to hydrolysis.

The substance does not benzoylate easily, and does not form a naphthalene sulphochloride derivative. On reduction with phosphorus and hydriodic acid in a sealed tube at 280° it did not give products suitable for analysis. It is unattacked by alkaline permanganate. Fuming nitric acid reacts with the substance to yield a bright yellow nitro-compound which is soluble in water, giving a yellow solution capable of dyeing silk. On the addition of alkali to the yellow solution, the colour changes to deep red. Oxidation of the substance with chromium trioxide and glacial acetic acid, and with potassium bichromate, gave rise to brown amorphous substances. With concentrated sulphuric acid no colour reaction is produced unless a trace of formaldehyde be added. The purple colour produced under these conditions is identical with that produced by the addition of a trace of glyoxylic acid to the suspension of the substance in water.

A. The original crystalline compound prepared from solutions of recrystallised tryptophane by using methods (*a*) and (*b*) was filtered from the mother liquor, washed well with water, alcohol and ether, dried and analysed.

(1) Dried at the ordinary temperature *in vacuo*:

0·1600 g.; 0·3575 g. CO_2, 0·0880 g. H_2O. C=60·94, H=6·11 %.
0·1230 g.; 12 c.c. moist nitrogen at 15° C. and 768 mm. N=11·42 %.
$C_{24}H_{26}O_5N_4 . H_2O$ requires C=61·49, H=6·04, N=11·96 %.

(2) Heated to a temperature of 155°:

0·1533 g.; 0·3730 g. CO_2, 0·0870 g. H_2O. C=66·35, H=6·36 %.
0·1710 g.; 0·4180 g. CO_2, 0·0860 g. H_2O. C=66·66, H=5·63 %.
0·1050 g.; 11·6 c.c. moist nitrogen at 15° C. and 768 mm. N=13·00 %.
0·2080 g.; 21·2 c.c. moist nitrogen at 7° C. and 776 mm. N=12·90 %.
$C_{24}H_{24}O_4N_4$ requires C=66·66, H=5·61, N=12·96 %.

B. The compound was twice dissolved in ten per cent. sodium carbonate solution and precipitated with dilute acetic acid. The constant melting point 324° was obtained. The crystalline precipitate was washed well with water, alcohol and ether, dried at various temperatures and analysed.

(1) Dried *in vacuo* at the ordinary temperature:

0·1200 g.; 0·2683 g. CO_2; 0·0645 g. H_2O. C=61·10, H=6·02 %.
0·1080 g.; 11·1 c.c. moist nitrogen at 22° C. and 767 mm. N=11·95 %.
$C_{24}H_{26}O_5N_4 . H_2O$ requires C=61·49, H=6·04, N=11·96 %.

(2) Dried at temperatures between 110° and 150°:

0·1187 g.; 0·2804 g. CO_2; 0·0645 g. H_2O. C=64·42, H=6·04 %.
0·1014 g.; 0·2395 g. CO_2; 0·0549 g. H_2O. C=64·41, H=6·05 %.
0·1252 g.; 0·2950 g. CO_2; 0·0672 g. H_2O. C=64·3, H=6·01 %.
0·1914 g.; 20·9 c.c. moist nitrogen at 18° C. and 748 mm. N=12·51 %.
0·1912 g.; 20 c.c. moist nitrogen at 11·5° C. and 757·2 mm. N=12·43 %.
$C_{24}H_{26}O_5N_4$ requires C=64·00, H=5·84, N=12·45 %.

(3) Heated to temperatures between 150° and 200°:

0·1037 g.; 0·2513 g. CO_2; 0·0545 g. H_2O. C=66·13, H=5·89 %.
$C_{24}H_{24}O_4N_4$ requires C=66·66, H=5·61, N=12·96 %.

It is thus evident that after solution of the ether oxidation substance in alkali and subsequent precipitation by acidification, the product precipitated has the same composition as when originally formed from the mother liquor.

It is of importance to note that the change in composition from $C_{24}H_{26}O_5N_4 . H_2O$ (*B* (1)) to $C_{24}H_{26}O_5N_4$ (*B* (2)) is of a reversible nature. That is to say, the ether oxidation substance previously heated to 140° will readily take up water again to give the monohydrate. But having effected the change

from $C_{24}H_{26}O_5N_4$ (*B* (2)) to $C_{24}H_{24}O_4N_4$ (*B* (3)), it is not possible to get the reverse reaction to take place. In other words, at temperatures above 150° the ether oxidation substance loses the elements of water as the result of some internal molecular rearrangement. The compound $C_{24}H_{24}O_4N_4$ does not undergo further change at temperatures from 200–220°, but it is decomposed at 225°, and carbon dioxide, ammonia and skatole are evolved.

II. *The condensation of formaldehyde and tryptophane.*

If a few cubic centimetres of formalin be added to a concentrated solution of pure tryptophane, and the reaction mixture be kept at a temperature of 38° for several hours, a crystalline derivative is deposited from the solution.

The compound melts and decomposes at 225–240°; it is slightly soluble in water and alcohol, insoluble in benzene and its homologues, amyl alcohol, chloroform, ethyl acetate and ethylene dibromide; it is of a slightly yellow colour which becomes intensified if the substance be kept at a temperature of 150° for a short time, or if it be left exposed to the air at ordinary room temperature for some days. On being boiled with water, dilute acids and weak alkalis, it is readily hydrolysed, formaldehyde is liberated and the ether oxidation product is formed. With concentrated sulphuric acid the substance gives a purple colour, which is intensified if the suspension of the substance in water be heated and cooled before adding the concentrated acid.

The condensation product was formed as follows:

(*a*) By the interaction of tryptophane and samples of formaldehyde obtained (1) from Merck's, (2) from Kahlbaum's, and (3) from Schering's formalin, (4) by distilling Merck's trioxymethylene in a current of hydrogen or nitrogen and collecting the issuing gases in air-free water.

(*b*) The formaldehyde vapours evolved in (4) were passed into a solution of tryptophane from which all air had been dispelled by a current of hydrogen.

In each experiment a crystalline derivative melting at 235–240° was formed.

On account of the hydrolysis which takes place with water, acids and alkalis, the substance could not be purified by being dissolved in alkalis and re-precipitated by acid. For purposes of analysis it was filtered from the mother liquor, washed well with water, alcohol and ether, and dried at different temperatures. Analyses of samples prepared at different times and dried at the same temperature indicated constancy of composition.

(1) Of the substance dried *in vacuo*:

0·1208 g.; 0·2537 g. CO_2; 0·0700 g. H_2O. C=57·30, H=6·49 %.
0·1135 g.; 0·2377 g. CO_2; 0·0658 g. H_2O. C=57·10, H=6·56 %.
0·1340 g.; 0·2820 g. CO_2; 0·0763 g. H_2O. C=57·33, H=6·38 %.
0·1175 g.; 10·7 c.c. moist nitrogen at 16° C. and 760 mm. N=10·81 %.
0·1512 g.; 14·4 c.c. moist nitrogen at 23° C. and 763·4 mm. N=10·95 %.
$C_{12}H_{12}O_2N_2 . 2H_2O$ requires C=57·14, H=6·35, N=11·11 %.

(2) Of the substance dried at temperatures between 110° and 130°:

0·1045 g.; 0·2355 g. CO_2; 0·0600 g. H_2O. C=61·45, H=6·43 %.
0·1135 g.; 0·2540 g. CO_2; 0·0635 g. H_2O. C=61·04, H=6·26 %.
0·1265 g.; 12·7 c.c. moist nitrogen at 25° C. and 765 mm. N=11·63 %.
$C_{12}H_{12}O_2N_2 . H_2O$ requires C=61·49, H=6·04, N=11·96 %.

(3) The substance was heated to 160° for ten minutes, but no analyses could be made owing to decomposition which began to take place; there was considerable carbonisation together with the elimination of skatole.

Analyses were made of the crystalline derivative formed by the hydrolysis of the formaldehyde product:

(1) *By water.* The derivative melted at 324°, and when mixed with some of the ether oxidation substance the melting point remained unchanged. The substance was dried at 110°.

0·1127 g.; 0·2678 g. CO_2; 0·0575 g. H_2O. C=64·80, H=5·72 %.
0·1055 g.; 0·2475 g. CO_2; 0·0518 g. H_2O. C=63·99, H=5·50 %.
0·1064 g.; 0·2520 g. CO_2; 0·0572 g. H_2O. C=64·50, H=5·98 %.
0·1340 g.; 14·8 c.c. moist nitrogen at 19° C. and 768·4 mm. N=12·94 %.

(2) *By Na_2CO_3 solution.* The derivative melted at 324°.

(*a*) Of the substance dried *in vacuo*:

0·1028 g.; 0·2290 g. CO_2; 0·0565 g. H_2O. C=60·80, H=6·16 %.

(*b*) Of the substance dried at 110°:

0·1000 g.; 0·2347 g. CO_2; 0·0525 g. H_2O. C=64·09, H=5·83 %.
0·1135 g.; 12·3 c.c. moist nitrogen at 21° C. and 768 mm. N=12·74 %.

These results are in accordance with the assumption from the melting point that the product of hydrolysis is the ether oxidation substance ($C_{24}H_{28}O_5N_4 . H_2O$ and $C_{24}H_{28}O_5N_4$).

It is thus evident that, although the product formed by the condensation of formaldehyde with tryptophane is not the same as the ether oxidation substance, yet there is a close connexion between these two which can be demonstrated by the fact that on hydrolysis the former is readily converted into the latter substance.

III. *The action of formaldehyde and hydrogen peroxide on tryptophane* gave rise to a crystalline compound which proved to be the formaldehyde product II, and not the ether oxidation substance.

IV. *The condensation of glyoxylic acid with tryptophane.*

To an aqueous solution of tryptophane was added glyoxylic acid in the proportion of one molecule of tryptophane to one molecule of glyoxylic acid. A crystalline substance was deposited from the solution.

The substance was slightly soluble in water and alcohol, insoluble in ether, petroleum ether, benzene and its homologues, amyl alcohol, ethyl acetate and ethylene dibromide. It is soluble in alkalis, moderately soluble in dilute acids, readily in concentrated acids. It is precipitated by the mercury sulphate reagent. It gives the glyoxylic reaction and also the bromine colour tests characteristic of tryptophane. It does not give any colour reaction with concentrated sulphuric acid; on the addition of a trace of formaldehyde or of glyoxylic acid solution, the glyoxylic colour is formed; while on the addition of a trace of hydrogen peroxide a claret colour is obtained.

The substance, whether separated from the mother liquors and repeatedly washed with water, alcohol and ether, or recrystallised from hot water and from alcohol, melted at 324°, and when mixed with some of the ether oxidation substance the melting point was unaffected. That the derivative under discussion was not the ether oxidation substance was shown by the fact that when dissolved in sodium carbonate solution and the solution acidified with acetic acid, the *acid sodium salt* was precipitated and not the original condensation product. Analyses of the original acid (*A*), and of the acid sodium salt (*B*) were made and the results obtained were in accordance with the formulae $C_{13}H_{12}O_4N_2$ for the acid and $C_{13}H_{11}O_4N_2Na.4H_2O$ for the sodium salt.

A. Analyses of the original product (m.p. 324°).

(1) Of the substance dried in vacuum desiccator:

0·1238 g.; 0·2693 g. CO_2; 0·0542 g. H_2O. C = 59·34, H = 4·89 %.
0·1858 g.; 18 c.c. moist nitrogen at 21° C. and 777·3 mm. N = 11·21 %.

(2) Dried at 150°:

0·1008 g.; 0·2193 g. CO_2; 0·0433 g. H_2O. C = 59·35, H = 4·82 %.
0·2820 g.; 26·2 c.c. moist nitrogen at 20° C. and 757·2 mm. N = 10·70 %.

(3) Heated to 200°:

0·1055 g.; 0·2287 g. CO_2; 0·0465 g. H_2O. C = 59·12, H = 4·99.
(1), (2) and (3). $C_{13}H_{12}O_4N_2$ requires C = 59·97, H = 4·66, N = 10·77 %.

(4) Heated to temperatures between 205° and 220° for ten minutes, carbon dioxide being evolved.

0·1184 g.; 0·2875 g. CO_2; 0·0575 g. H_2O. C = 66·23, H = 5·44 %.
0·1525 g.; 16·8 c.c. moist nitrogen at 14·5° C. and 755·3 mm. N = 12·94 %.
$C_{24}H_{24}O_4N_4$ (ether oxidation substance) requires C = 66·66, H = 5·61, N = 12·96 %.

It is evident from the above analyses that at temperatures between 15° and 200° the glyoxylic condensation product has a constant composition; at 220° it is converted, with loss of carbon dioxide, into the ether oxidation substance.

(5) If the glyoxylic compound be heated for a short time at a temperature of 225–230° it decomposes, yielding carbon dioxide, ammonia and skatole (cf. I, p. 105).

B. The acid sodium salt (see above) was twice recrystallised from water and analysed.

0·3735 g.; 26·1 c.c. moist nitrogen at 20·8° C. and 757·3 mm. N = 8·08 %.
0·1289 g.; 9 c.c. moist nitrogen at 21·5° C. and 761·9 mm. N = 8·02 %.
0·2048 g.; ·0406 g. Na_2SO_4. Na = 6·42 %.
0·3700 g.; 0·0562 g. Na_2CO_3 and 0·0762 g. Na_2SO_4. Na = 6·59 and 6·67 %.
0·8122 g.; 0·1542 g. Na_2SO_4. Na = 6·15 %.

From these determinations the molecular weight of the substance = 355.

0·6074 g.; 0·4890 g. anhydrous residue,
∴ in the acid salt there are 3·84 mols. water.
0·1033 g.; 0·0528 g. H_2O; 0·1612 g. CO_2.
Putting in the correction for the Na_2CO_3 remaining in the boat (0·0150 g.) } C = 44·20, H = 5·72 %.
$C_{13}H_{11}O_4N_2Na.4H_2O$ requires N = 7·91, C = 44·07 %.
H = 5·37, Na = 6·50 %.
M.W. 354·3.

From these results it is obvious that the glyoxylic condensation product and the ether oxidation substance are two distinct compounds, in spite of the fact that the compounds when taken separately and also when mixed together have the same melting point, viz. 324°. It is obvious that the glyoxylic compound readily changes to the ether oxidation substance on being heated to a temperature of 205°. (The ether oxidation substance is not affected under the same conditions.) At 225° both are decomposed into carbon dioxide, ammonia, skatole, etc.

There is thus an interesting connexion between the formaldehyde compound, the glyoxylic condensation product, and the ether oxidation substance. The last-named substance can be produced from the formaldehyde product by hydrolysis, and from the glyoxylic condensation product by the application of heat.

It is probable from the following reasons that these compounds described under I, II and IV are formed by the simple process of condensation between the $-NH_2$ group of tryptophane and the $-CHO$ group of the reacting aldehydes:

1. The ease with which the reactions take place (the presence of a condensing agent is unessential).

2. The failure to produce naphthalene sulphochloride derivatives points to the substitution of the hydrogen of the amino-groups.

3. The fact that the compounds so obtained are colourless points to the condensation having taken place as a result of the activity of the amino- and not the imino-group (*post*, p. 112).

4. Corresponding compounds could not be obtained with indole, skatole, indoleacetic and indolepropionic acids, i.e. with indole derivatives in which there is no *amino*-group.

For reaction to take place with these compounds, the presence of a condensing agent is necessary, and the resulting products are intensely coloured (*post*, p. 112).

Analyses of the three compounds I, II, IV are in accordance with the following formulae:

(*a*)	The formaldehyde product	$C_{12}H_{12}O_2N_2 . 2H_2O$.
(*b*)	The glyoxylic product...	$C_{13}H_{12}O_4N_2$.
	„ „ „ above 205° ...	$C_{24}H_{24}O_4N_4$.
(*c*)	The ether oxidation substance ...	$C_{24}H_{26}O_5N_4$.
	„ „ „ „ above 150°	$C_{24}H_{24}O_4N_4$.

The mechanism involved in the production of these three substances may be briefly described as follows:

I. Since the *ether oxidation product* is in no way an oxidation product of tryptophane, but is closely connected with the formaldehyde condensation product, it is suggested that in the formation of the ether oxidation substance nascent formaldehyde is the active principle (p. 102, I c).

$$R.CH_2.CH(NH_2).COOH + 2HCHO\ (\text{nascent}) \rightarrow R.CH_2.CH(COOH).NH.CH_2.O.CH_2.NH.CH(HOOC).CH_2R$$

(where R represents the indole nucleus C_8H_6N).

On heating this substance to a temperature of 150–200°, molecular water and not water of crystallisation is lost. After such a process the composition of the substance corresponds to the formula $C_{24}H_{24}O_4N_4$, and can be represented graphically as

$$\begin{matrix} COOH \\ R.CH_2 \end{matrix}\!\!>\!CH.NH.CH=CH.NH.CH\!<\!\begin{matrix} COOH \\ CH_2.R \end{matrix}$$

II. *The formaldehyde condensation product* is formed as follows:

$$R.CH_2.CH(NH_2).COOH + HCHO = R.CH_2.CH\begin{cases}COOH\\N:CH_2\end{cases} + H_2O$$

III. The *glyoxylic condensation product* is produced in a similar manner:

$$R.CH_2.CH(NH_2).COOH + COOH.CHO = R.CH_2.CH\begin{cases}COOH\\N:CH.COOH\end{cases} + H_2O$$

It has been shown that the ether oxidation substance is readily formed (*a*) from the formaldehyde condensation product by hydrolysis, (*b*) from the glyoxylic condensation product by the application of heat. For the former process (*a*) there are two possibilities to be considered, viz.:

(1) That during hydrolysis the molecules are linked together by means of the addition of the elements of water, thus:

$$2R.CH_2.CH\begin{cases}COOH\\N:CH_2\end{cases} + H_2O = R.CH_2.\overset{COOH}{CH}.NH.CH_2.O.CH_2.NH.\overset{COOH}{CH}.CH_2.R$$

(2) That during the hydrolysis tryptophane and nascent formaldehyde are re-formed, and that these react as in the ether oxidation process to form the substance $C_{24}H_{26}O_5N_4$.

(*b*) In the case of the production of the substance $C_{24}H_{24}O_4N_2$ from the glyoxylic product by the application of heat, it is evident that two molecules of the latter each lose a molecule of carbon dioxide, and at the temperature at which the reaction takes place condensation of these two molecules occurs, accompanied by a rearrangement of the bonds into the more stable configuration of the ether oxidation substance.

$$2R.CH_2.CH(COOH).N:CH.COOH = R.CH_2.CH(COOH).NH.CH:CH.NH.CH(COOH).CH_2.R + 2CO_2.$$

A study of the properties of these three compounds throws some light on the mechanism of the Adamkiewicz reaction: a discussion of this point will be dealt with in the following paper [Homer, 1913, p. 117, Table I].

Section B.

The condensation of glyoxal with tryptophane.

If an aqueous solution of tryptophane heated to a temperature of 38° be treated with the calculated quantity of glyoxal in the presence of hydrogen peroxide, a crystalline chocolate-coloured deposit slowly forms.

This coloured substance is of an acidic nature and is slightly soluble in water and in alcohol. It dissolves in alkalis, and is moderately soluble in

dilute acids, and with the exception of acetic acid readily soluble in concentrated acids. It is precipitated by the mercury sulphate reagent. With concentrated sulphuric acid it gives a brown colour which in the presence of hydrogen peroxide changes to claret. In the presence of a trace of formaldehyde, a brown colour is produced which is changed to the colour of bromine on the addition of an oxidising agent.

Analyses of the substance gave the following results:

(1) Of the substance recrystallised from water and

(*a*) Dried at 110°:

0·1030 g.; 0·2325 g. CO_2; 0·0463 g. H_2O. C=61·60, H=5·04 %.
0·1223 g.; 0·2780 g. CO_2; 0·0560 g. H_2O. C=61·99, H=5·13 %.
0·1025 g.; 0·2346 g. CO_2; 0·0485 g. H_2O. C=62·40, H=5·25 %.
0·2268 g.; 21·6 c.c. moist nitrogen at 24° C. and 767·5 mm. N=10·9 %.
$C_{26}H_{22}O_6N_4.H_2O$ requires C=61·90, H=4·81, N=11·1 %.

(*b*) Heated to 130°:

0·1006 g.; 0·2360 g. CO_2; 0·0412 g. H_2O. C=64·00, H=4·62 %.
0·1080 g.; 10·7 c.c. moist nitrogen at 21° C. and 768 mm. N=11·51 %.

(2) After solution in Na_2CO_3 and precipitation by acetic acid:

0·1430 g.; 0·0565 g. H_2O; 0·3403 g. CO_2. C=64·90, H=4·43 %.
0·1060 g.; 10·2 c.c. moist nitrogen at 20° C. and 769 mm. N=11·24 %.

(3) Crystallised from alcohol and heated to 110°:

0·1017 g.; ·0441 g. H_2O; ·2390 g. CO_2. C=64·15, H=4·86 %.
0·1290 g.; 12·5 c.c. moist nitrogen at 19° C. and 750·5 mm. N=11·1 %.

(4) Crystallised from pyridine:

0·1060 g.; ·0441 g. H_2O; 0·2505 g. CO_2. C=64·45, H=4·63 %.
Ib. 2, 3, 4:—$C_{26}H_{22}O_6N_4$ requires C=64·2, H=4·53, N=11·5 %.

(5) Heated to 160°:

0·1006 g.; 0·0410 g. H_2O; 0·2459 g. CO_2. C=66·66, H=4·53 %.
0·1150 g.; 0·0460 g. H_2O; 0·2830 g. CO_2. C=67·07, H=4·46 %.
0·1890 g.; 19·6 c.c. moist nitrogen at 16° C. and 749·6 mm. N=12·01 %.
$C_{26}H_{20}O_5N_4$ requires C=66·66, H=4·32, N=11·97 %.

Taking into consideration the necessity for the presence of an oxidising agent, and also the fact that the substance produced is intensely coloured, it is highly probable that in this reaction, besides the simple aldehyde condensation occurring in the reactions described in Section A, there has also been elimination of hydrogen accompanied by complex ring formation.

Possible equation:

$$2C_{11}H_{12}O_2N_2 + 2(CHO)_2 + O = C_{26}H_{22}O_6N_4.H_2O + 2H_2O.$$

Any attempt to ascertain the constitution of this substance has been postponed for the present.

Section C.

The Products formed by the Condensation of Aldehydes with the Imino-Group of Indole Derivatives.

I. *The action of formaldehyde on indole derivatives in presence of a condensing agent.*

In connexion with the work on the condensation of formaldehyde with tryptophane, an attempt was made to prepare corresponding derivatives with indole, skatole, indolepropionic and indoleacetic acids, but without success. However, it is well known that under suitable conditions, in the presence of concentrated sulphuric acid, a solution of formaldehyde reacts with the above-mentioned compounds to yield coloured substances. Concentrated sulphuric acid being known to be both an oxidising and a condensing agent, the question arose as to whether the colour effect was due to a process of simple condensation between formaldehyde and the above-mentioned substances, which have an imino-group in common, or was in any way dependent upon the oxidising power of the acid.

In order to test this suggestion, it was thought advisable to avoid the use of sulphuric acid and attempts were made to form condensation products of indole derivatives with trioxymethylene, using zinc chloride and alcohol saturated with hydrochloric acid gas as the condensing agents (with these condensing agents an aqueous solution of formaldehyde could not be used).

II. *Derivatives formed by the interaction of trioxymethylene and indole derivatives, using zinc chloride as the condensing agent.*

Indole, and indoleacetic acid reacted readily at temperatures below 100° to produce purple-coloured products. Skatole and indolepropionic acid under the same conditions gave brown products. Owing to scarcity of material, the products from indoleacetic and indolepropionic acids could not be analysed. The indole and skatole derivatives were investigated.

Tryptophane treated in the same way did not react until heated to a much higher temperature (160°); the resulting product was of a *deep brown* colour. The reaction was accompanied by a certain amount of decomposition of tryptophane. As it was impossible to free the product of the reaction from the results of carbonisation, no further investigation of this substance has been made.

Preparation of the trioxymethylene derivatives of skatole and indole. One part by weight of indole or skatole was mixed with the same weight of trioxymethylene and ground together with one part by weight of freshly powdered and freshly ignited zinc chloride. The process of grinding the reaction mixture gave rise in the former case to the formation of a purple colour. The reaction mixture was heated on a water-bath to a temperature of 80° for half an hour, and then transferred to an oil bath and the temperature raised to 150° for a few minutes. On cooling, the coloured mixture was treated with hydrochloric acid solution (one part concentrated acid to one part water) and allowed to stand for some hours. The residue was in each case repeatedly boiled with dilute hydrochloric acid, and then with water until free from formaldehyde and hydrochloric acid. The coloured substance was finally washed with water, alcohol and ether, dried and analysed.

It was impossible to purify the products further on account of their insolubility in all liquids other than concentrated sulphuric acid. Constancy of composition was denoted by analyses of samples prepared at different times and under slightly different conditions as regards the proportion of zinc chloride used. It is interesting to note that the indole compound is of a purplish colour, the skatole compound is brown. They are both insoluble in ordinary solvents, in alkalis and in acids with the exception of concentrated sulphuric acid, in which they are appreciably soluble, and concentrated hydrochloric acid in which they are slightly soluble. In concentrated sulphuric acid the indole derivative gives a reddish violet colour which on dilution becomes bluish and finally disappears owing to the decreasing solubility of the substance as the acid becomes more dilute. The skatole compound under the same conditions gives rise to a brown colour.

(1) Analyses of the indole derivative prepared on different occasions were made[1].

A. 0·1026 g.; 0·0475 g. H_2O; 0·2702 g. CO_2. C = 71·85, H = 5·20 %.
B. 0·1057 g.; 0·0518 g. H_2O; 0·2783 g. CO_2. C = 71·80, H = 5·49 %.

A. 0·1325 g.; 9·4 c.c. moist nitrogen at 14° C. and 753·5 mm. N = 8·32 %.
0·1500 g.; 11·0 c.c. moist nitrogen at 15·5° C. and 747·3 mm. N = 8·34 %.
B. 0·2074 g.; 14·2 c.c. moist nitrogen at 13° C. and 755·3 mm. N = 8·07 %.

Analysis of the indole derivative after being heated to 205° for fifteen minutes gave the following result:

0·1045 g.; 0·0502 g. H_2O; 0·2750 g. CO_2. C = 71·78, H = 5·38 %.

Hence the compound is stable to heat at temperatures not exceeding 205°.

$C_{21}H_{20}O_3N_2$ requires C = 72·41, H = 5·74, N = 8·04 %.

[1] These compounds are extremely difficult to burn and this probably accounts for the slightly low values obtained.

(2) Analyses of the skatole derivative gave the following results[1]:

0·1161 g.; 0·0658 g. H_2O; 0·3120 g. CO_2. C = 73·30, H = 6·35 %.
0·1083 g.; 0·0575 g. H_2O; 0·2910 g. CO_2. C = 73·30, H = 5·94 %.
0·1685 g.; 9·8 c.c. moist nitrogen at 13·5° C. and 758·5 mm. N = 7·13 %.
0·1820 g.; 10·8 c.c. moist nitrogen at 11·5° C. and 765 mm. N = 7·15 %.
$C_{23}H_{24}O_3N_2$ requires C = 73·40, H = 6·38, N = 7·42 %.

The condensations may therefore be represented by the equations:

$$2C_8H_7N + 5HCHO = C_{21}H_{20}O_3N_2 + 2H_2O$$

$$2C_9H_9N + 5HCHO = C_{23}H_{24}O_3N_2 + 2H_2O.$$

It is thus obvious that in the presence of a condensing agent trioxymethylene reacts with the imino-group of the indole nucleus to form coloured bodies.

III. *Derivatives formed by the interaction of trioxymethylene and indole derivatives, using a solution of hydrochloric acid gas in alcohol as the condensing agent.*

A description of the condensation products with indole, skatole and tryptophane formed under these conditions will form the subject-matter of a future paper. It may be stated here that they are intensely coloured substances, and that the indole derivative is the only one of the three which will give a purple colour with concentrated sulphuric acid.

The derivatives formed by the action of trioxymethylene on indole derivatives in the presence of a condensing agent, whether it be concentrated sulphuric acid, or zinc chloride, or an alcoholic solution of hydrochloric acid gas, are all characterised by their insolubility in solvents other than concentrated mineral acids.

A discussion as to the significance of activity of the *imino*-group of indole derivatives in the production of the specific colour reaction known as the Adamkiewicz reaction, will be found in the paper following this [Homer, 1913, p. 116].

During the course of the work under consideration it has been noticed that tryptophane itself under certain conditions of oxidation gives rise in the process of time to the formation of pigments having definite absorption bands. The properties both chemical and physical of these pigments, and of those formed from normal breakdown products of tryptophane, are being investigated, and will form the subject of a future paper. At this

[1] These compounds are extremely difficult to burn and this probably accounts for the slightly low values obtained.

point it may be observed that in nearly every case these substances are themselves brown in colour, and their solutions in organic solvents are also brown, but the presence of acid changes the colour from brown to purple.

SUMMARY.

1. Tryptophane by virtue of its $-NH_2$ group reacts directly with nascent formaldehyde, formaldehyde and glyoxylic acid to form colourless crystalline compounds.

2. Glyoxal will react with tryptophane in the presence of an oxidising agent to form an intensely coloured compound.

3. Indole derivatives, by virtue of the $-NH$ group in the nucleus, will react with formaldehyde and trioxymethylene *in the presence of a condensing agent* to form substances of intense colour and marked insolubility in ordinary solvents other than concentrated mineral acids.

In conclusion, the author wishes to thank Dr Hopkins for his unfailing interest in the work and for the valuable advice given during the progress of the investigation.

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XI. ON THE COLOUR REACTIONS OF CERTAIN INDOLE DERIVATIVES, AND THEIR SIGNIFICANCE WITH REGARD TO THE GLYOXYLIC REACTION.

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(*Received November 26th, 1912.*)

In the preceding paper [Homer, 1913] the preparation and properties of several new indole derivatives have been described in detail.

The condensation products formed between tryptophane and nascent formaldehyde, formaldehyde and glyoxylic acid respectively in the absence of a condensing agent, are colourless crystalline compounds. The reaction takes place between the $-CHO$ group of the aldehyde and the $-NH_2$ group of tryptophane.

In the presence of condensing agents, such as zinc chloride or an alcoholic solution of hydrochloric acid gas, indole derivatives and pyrrole, by virtue of the $-NH$ group in the nucleus, combine with trioxymethylene to form coloured substances; in the presence of concentrated sulphuric acid a solution of formaldehyde reacts with indole and pyrrole derivatives to give colour reactions.

In the case of the condensation of glyoxal with tryptophane, the presence of an oxidising agent is necessary, and the resulting product, which is coloured, no longer gives the glyoxylic reaction.

The results of an investigation of the colour reactions of the above-mentioned substances have been embodied in the following tables, and afford special interest with regard to the mechanism of (*a*) the Adamkiewicz reaction for the detection of proteins and (*b*) Hopkins and Cole's glyoxylic reaction.

Section I.

(In the following reactions, air-free water has been used in every case.)

TABLE I. *Colour reactions produced by the action of pure concentrated sulphuric acid on the formaldehyde, and glyoxylic acid condensation products of tryptophane, on the ether oxidation substance and on tryptophane.*

Action of concentrated sulphuric acid	Tryptophane	Formaldehyde product	Glyoxylic condensation product	Ether oxidation substance
A. On the solid:				
i. In the cold	None	Purple[1]	Yellow	None
ii. On warming	Slight darkening. Finally charring	Colour intensified. Finally charring	Colour intensified. Finally charring	Slight purple tint
B. On the solid suspended in water:				
i. Directly treated with the acid	None	Purple	Yellow	None
ii. Heated to 90°, cooled and then treated with the acid	None	Purple colour intensified	Yellow	None
iii. In the presence of one drop of a 1/200 solution of formalin in water	Brown	Purple colour intensified	Purple	Purple
iv. In the presence of a trace of glyoxylic acid	Purple	Purple	Purple	Purple
v. In the presence of a trace of ferric chloride	Orange red	Very intense purple	Brown	Green
vi. In the presence of a trace of hydrogen peroxide	Orange red	Brown	Claret	Green

[1] The term "purple" has been used for colours that are red with a tinge of blue in them, or vice versâ.

TABLE II. *The colour reactions produced by the action of concentrated sulphuric acid on pyrrole and indole derivatives in presence of glyoxal.*

Action of concentrated sulphuric acid	Pyrrole	Indole	Skatole	Indole-propionic acid	Tryptophane
In the presence of:					
i. A trace of glyoxal ...	Reddish brown	Indian red	Burnt Sienna	Reddish brown	Claret
to which hasb een added a trace of:					
(*a*) Ferric chloride ...	Greenish tint	Greenish tint	Reddish brown	Deep orange which rapidly changes to purple madder tint	Claret colour intensified
(*b*) Hydrogen peroxide...					
ii. An infinitesimal trace of glyoxal	Yellow brown	Very delicate shade of Indian red	Reddish brown	Yellowish brown with a tinge of red	Russet
to which has been added a trace of:					
(*a*) Ferric chloride ...	Intense blue changing to bluish purple	Green	Reddish brown	Orange red which rapidly changes to a purple madder	Russet colour intensified
(*b*) Hydrogen peroxide...	Green	Green	Magenta		

TABLE III. *The colour reactions produced by the action of pure concentrated sulphuric acid on pyrrole and various indole derivatives in the presence of* (A) *formaldehyde, and* (B) *glyoxylic acid.*

Action of concentrated sulphuric acid	Pyrrole	Indole	Skatole	Indole-propionic acid	Tryptophane	Glyoxylic condensation product	Ether oxidation substance
A. i. In the presence of 1 drop of a solution of 1 c.c. of formalin in 10 c.c. of water	Red brown	Purple	Brown	Brown	Brown	Purple	Purple
ii. In the presence of 1 drop of a 1/200 solution of formalin in water	Red brown	Purple	Brown	Brown	Brown	Purple	Purple
to which has been added a trace of an oxidising agent	Green	Purple	Purple	Purple	Purple	Purple	Purple
iii. In the presence of 1 drop of a 1/2000 solution of formalin in water	Yellowish brown	Purple	Purple	Purple	Purple	Purple	Purple
iv. In the presence of 1 drop of a solution of formaldehyde made by dissolving 1 part of trioxymethylene in 1000 parts boiling water	Red brown	Purple	Brown	Brown	Brown	Purple	Purple
v. In presence of 1 drop of the solution used in iv, diluted ten times	Yellowish brown	Purple	Purple	Purple	Purple	Purple	Purple
B. In the presence of glyoxylic acid	Red brown	Purple	Purple	Purple	Purple	Purple	Purple

TABLE IV. *The colour reactions produced by the direct action of pure concentrated sulphuric acid on the trioxymethylene condensation derivatives of indole, skatole and tryptophane.*

(*a*) Formed in the presence of zinc chloride as condensing agent.

(*b*) Formed in the presence of an alcoholic solution of hydrochloric acid gas.

Action of concentrated sulphuric acid	Indole derivative	Skatole derivative	Tryptophane derivative
(*a*) On the solid	Purple	Brown	Brown
(*b*) On the solid	Purple	Brown	Brown

Tables V*a* and V*b* illustrate the masking effect due to the presence of trioxymethylene.

TABLE V *a*. *Colour reactions produced by the action of pure concentrated sulphuric acid on aqueous solutions or suspensions of indole derivatives.*

Action of pure concentrated sulphuric acid	Indole	Skatole	Indole-propionic acid	Trypto-phane	Glyoxylic condensation product and ether oxidation substance
i. In the presence of 1 drop of a 1/200 solution of formalin in water	Purple	Brown	Brown	Brown	Purple
to which has been added an oxidising agent	Purple	Purple	Purple	Purple	Purple
ii. In the presence of a trace of trioxymethylene	Purple	Brown	Brown	Brown	Purple
iii. In the presence of 1 drop of a 1/200 solution of formalin in water, an oxidising agent, and a trace of trioxymethylene, cf. i (above)	Purple	Brown	Momentary appearance of purple colour, immediately masked by brown colour	Brown	Purple

TABLE V *b*. *Colour reactions produced by the action of concentrated sulphuric acid, in which has been dissolved a trace of trioxymethylene, on aqueous solutions or suspensions of indole derivatives.*

Action of concentrated sulphuric acid, containing a trace of trioxymethylene	Indole	Skatole	Indole-propionic acid	Trypto-phane	Glyoxylic condensation product and ether oxidation substance
i. Directly on the aqueous solution or suspension	Purple	Brown	Brown	Brown	Purple
ii. In the presence of an oxidising agent	Purple	Brown	Momentary appearance of purple, very soon masked by predominating brown	Brown	Purple
iii. In the presence of an oxidising agent and 1 drop of a 1/200 solution of formalin in water	Purple	Brown	Momentary appearance of purple, very soon masked by predominating brown	Brown	Purple

Adamkiewicz [1874] demonstrated that a violet colour is produced by the action of concentrated sulphuric acid on an acetic acid solution of protein. Hopkins and Cole [1900; 1902] showed that the protein reacts by virtue of its tryptophane groupings, and that *pure* acetic acid does not give the colour test. As a result of their investigations they were led to the conclusion that the reactive impurity in the acetic acid was glyoxylic acid.

Therefore, in the Adamkiewicz reaction, Hopkins and Cole regarded

glyoxylic acid as the substance essential to the formation of the characteristic violet colour. Rosenheim [1906] however, points out that the colour produced by the action of formaldehyde on proteins in presence of sulphuric acid and oxidising agents [Voisenet 1905] is identical with that produced in the Adamkiewicz reaction. He criticises Hopkins and Cole's view as to the importance of glyoxylic acid *per se*; he suggests that even with glyoxylic acid an oxidising agent is essential, and that although the latter may not have been added, yet the glyoxylic acid itself, however carefully prepared, is always contaminated with sufficient hydrogen peroxide for the reaction to take place. On the other hand Dakin [1909], noticing that the colours produced by the action of glyoxylic acid and formaldehyde are identical, suggests that in the case of formaldehyde, under the influence of acids, an aldol condensation and subsequent oxidation to glyoxylic acid take place.

From Table I it is obvious that formaldehyde *per se* plays an important part in the colour reaction, since of the three condensation products, the only one which will give the colour without the addition of glyoxylic acid or formaldehyde, is the formaldehyde condensation product. Further it has been shown that this product is readily hydrolysed with liberation of formaldehyde; at the line of junction of concentrated acid and water such hydrolysis would take place. A proof of this assumption lies in the observation that after boiling this product with water and so ensuring hydrolysis, the colour reaction is still more marked (Table I, B ii).

Were the essential factor glyoxylic acid *per se*, the glyoxylic condensation product ought to give the colour reaction with sulphuric acid, but it will not do so unless formaldehyde or more glyoxylic acid be added. Now glyoxylic acid itself is decomposed by sulphuric acid with the formation of carbon dioxide and formaldehyde[1]. It therefore seems reasonable to assume that glyoxylic acid is able to take part in the colour reaction by virtue of its decomposition into formaldehyde, and not, as Rosenheim suggests, because of contamination with hydrogen peroxide. The fact that the colour reaction is not given by glyoxal (Table II) and glycollic aldehyde, neither of which gives formaldehyde on treatment with acid, is in support of this view.

On the other hand, if formaldehyde be the essential factor, then some explanation must be offered to account for the generally acknowledged uncertainty of the colour reaction when that reagent is used, whereas with glyoxylic acid there is no difficulty experienced in carrying out the test for indole derivatives.

[1] A small quantity of glyoxylic acid was heated with 5 c.c. of water and 5 c.c. of concentrated sulphuric acid. The gases evolved were found to contain formaldehyde and carbon dioxide.

According to Hopkins and Cole, Dakin, and Rosenheim, the presence of an oxidising agent is necessary for the Adamkiewicz reaction to be carried out with formaldehyde.

In the present investigation samples of pure sulphuric acid (Kahlbaum and others) were specially procured and carefully selected so that they did not induce a purple colour with tryptophane and a 1/500 solution of formalin in air-free water. The addition of a trace of ferric chloride solution to the reacting liquids induced the purple colour to appear. Samples of sulphuric acid contaminated with a trace of an oxidising agent caused the appearance of the purple colour with the use of a 1/100, and in some cases a 1/10 solution of formalin in water.

A study of Table III shows that, using a 1/200 solution of formalin in water, without the addition of an oxidising agent, indole, indolealdehyde, indoleacetic acid, and the condensation products of tryptophane gave a purple colour, skatole, tryptophane and indolepropionic acid gave brown colourations. The addition of a trace of an oxidising agent to the latter was necessary for the production of the purple colour. In this connexion the following observations have been made:

1. In applying the formaldehyde test to tryptophane and skatole, if previous to the addition of the above-mentioned formaldehyde solution a trace of indolealdehyde be added, then the presence of the concentrated sulphuric acid will induce the formation of the characteristic purple colour, even though no oxidising agent has been added. This experiment shows that for the production of the purple colour the indole derivative and not the formaldehyde is attacked by the oxidising agent.

2. If to an aqueous solution of tryptophane one or two drops of formalin be added, the liquid boiled, cooled, diluted and treated with concentrated sulphuric acid, then the characteristic purple is produced, but is soon masked by the brown colouration usually noticed.

In this experiment the presence of one of the condensation products of tryptophane which gives a purple colour with formaldehyde has been assured.

In order to ascertain whether this purple colour reaction was due to an aldol condensation and oxidation of formaldehyde to glyoxylic acid [Dakin, 1909], the formalin itself was boiled, cooled, diluted to 1/200 and added to a cold aqueous solution of tryptophane. There was no production of a purple colour.

It will be seen from Table III, A iii and v that if the formaldehyde be diluted to the order of 1 part to 2000 or 20,000 parts of water, then the

purple colour is formed with skatole, tryptophane and indolepropionic acid without the addition of an oxidising agent. This result was obtained with Kahlbaum's and Merck's formalin, and also with a solution of formaldehyde made by dissolving trioxymethylene in boiling water which had been previously boiled for some time to expel air. The substances to be tested were dissolved in air-free water. Even with such large dilution, there is in the case of tryptophane a certain amount of brown colouration produced, but not sufficient to mask the purple colour. As the concentration of the formalin is increased, so the brown colour preponderates and masks the purple.

These experiments, taken in conjunction with the fact that the formaldehyde condensation product is the only one which gives the Adamkiewicz reaction without the addition of glyoxylic acid or formaldehyde, seem to be conclusive as to the importance of formaldehyde *per se* as a factor in this reaction. But at the same time, in performing the Adamkiewicz colour reaction the fact that concentrated sulphuric acid causes the condensation of formaldehyde to trioxymethylene must be taken into consideration. In the previous paper it has been shown that trioxymethylene, in the presence of a condensing agent, reacts with indole and indoleacetic acid to give purple derivatives; with skatole, indolepropionic acid and tryptophane to give brown coloured substances. In the former cases, therefore, the secondary action with trioxymethylene will not interfere with the purple colour formation, but in the latter cases the brown colour due to the formation of trioxymethylene derivatives will preponderate. The correctness of this assumption is demonstrated by the results described in Tables V *a* and V *b*. It will be noticed that a trace of trioxymethylene interferes with the appearance of the purple colour which would normally be produced under the conditions of the experiment (V *a* iii and V *b* iii). The masking effect due to the formation of trioxymethylene by the action of concentrated sulphuric acid on the formaldehyde may be obviated:

1. By using the formaldehyde solution excessively dilute, whereby the formation of trioxymethylene is delayed, and the brown colouration due to the condensation of certain indole derivatives with trioxymethylene does not interfere with the Adamkiewicz test.

2. By the addition of a trace of an oxidising agent, whereby the formation of an indole derivative, which will give the colour test, is induced.

3. In the case of tryptophane, by ensuring the previous formation of one of the three condensation products described above (p. 121).

4. By the use of glyoxylic acid as the source of formaldehyde instead

of formaldehyde itself (Hopkins and Cole's modification of the Adamkiewicz reaction).

The author suggests that at the line of junction of the concentrated sulphuric acid and the aqueous layer containing a trace of glyoxylic acid and the indole derivative, decomposition of the glyoxylic acid takes place with the liberation of formaldehyde. The vivid colour reaction may be assigned to one of the following causes:

1. The amount of formaldehyde produced under the conditions of the experiment is never more than a trace, and the corresponding trioxymethylene formation does not interfere with the colour reaction (Table III).

2. The formaldehyde is liberated in the nascent state, and is therefore intensely reactive.

Either of these explanations can be offered to account for the vivid colour reaction when the formaldehyde condensation product of tryptophane is acted upon by concentrated sulphuric acid without the previous addition of formaldehyde, glyoxylic acid or an oxidising agent. But that the nascent condition of the formaldehyde is not essential to the colour reaction is deduced from the fact that the addition of a 1/10 solution of formalin in water to the ether oxidation substance or the glyoxylic condensation product gives rise in the presence of concentrated sulphuric acid, to the formation of a purple colour.

3. In the glyoxylic reaction with tryptophane, the colour reaction is probably influenced by the action of the formaldehyde, liberated from the glyoxylic acid, on the glyoxylic condensation product formed with tryptophane.

Section II.

The following experiments show that the colour reaction induced by the action of concentrated sulphuric acid on solutions of indole derivatives to which formaldehyde and an oxidising agent have been added, is of a much more complex nature than when formaldehyde alone is used.

In Section I (p. 121) it was demonstrated that the so-called "glyoxylic" colour reactions could be obtained by the action of concentrated sulphuric acid on solutions of tryptophane and skatole to which had been added traces of indolealdehyde and formaldehyde. Therefore in the Adamkiewicz test the part played by the oxidising agent is to convert the tryptophane and skatole into some indole derivative which will react with formaldehyde to form compounds capable of giving purple colour reactions with sulphuric acid and not to oxidise the formaldehyde as Dakin suggests.

A study of the colour reactions of indolealdehyde was made:

Colour reactions induced by the action of concentrated sulphuric acid on solutions containing:

		Colour produced.
(1)	Indolealdehyde.	Pink. Colour becomes more intense on warming [Ellinger and Flamand, 1909].
(2)	Indolealdehyde + one drop of a 1/100 solution of formalin.	Reddish purple. The addition of an oxidising agent causes the colour to become more blue.
(3)	Indolealdehyde + trace of trioxymethylene.	,, ,,
(4)	Indolealdehyde + a trace of an oxidising agent.	Bluish magenta.
(5)	Indolealdehyde + tryptophane.	Orange red colour.
(6)	Indolealdehyde + tryptophane + a trace of formaldehyde (1/100).	"Glyoxylic" colour at the line of junction of the acid and aqueous solution. Above this is seen an orange red colour.
(7)	Indolealdehyde + tryptophane + an oxidising agent.	Magenta.
(8)	Indolealdehyde + tryptophane + a trace of trioxymethylene.	"Glyoxylic" colour as in (6) but it is rapidly masked by the brown colour characteristic of the action of trioxymethylene on tryptophane.
(9)	Indolealdehyde + skatole.	Reddish pink colour.
(10)	Indolealdehyde + skatole + a trace of formaldehyde (1/100).	At the junction of the acid and solution a reddish purple colour is obtained (as in (11)). Above this layer the colour is reddish pink.
(11)	Skatole + a trace of an oxidising agent + a trace of formaldehyde (1/100).	Reddish purple.

If the indolealdehyde in (10) be present in excess then the colour produced is practically that produced in (9), the purple being masked.

It is obvious from these experiments that where colour reactions are produced from tryptophane in the presence of an oxidising agent the secondary reactions with indolealdehyde have to be considered, and in these cases the actual shade of colour will depend upon the conditions of the experiment.

From (6) it will be seen that the "glyoxylic" colour is produced by the action of formaldehyde (*without the addition of an oxidising agent*) on the mixture of tryptophane and indolealdehyde. It may be that the indolealdehyde reacts with the amino-group of tryptophane to form a substance which then combines with formaldehyde as did the three condensation products (Section I, Table I).

An inspection of reactions (4) and (7) shows that in the presence of an oxidising agent concentrated sulphuric acid causes indolealdehyde to condense with more indolealdehyde (4) or with tryptophane (7) to form substances giving characteristic colour reactions which are not those of the "glyoxylic" reaction.

Although the chemical reactions involved in the actual process of the purple colour formation are at present unsolved yet it is clear that the condensation of indole nuclei is an essential feature of the reaction. In the case of those compounds which give the purple colour directly with formaldehyde the condensation takes place through the agency of the formaldehyde alone. But for those substances for which, on account of interference with the colour reaction due to the formation of trioxymethylene, an oxidising agent is used, the reaction also involves the condensation of indolealdehyde with the original indole derivative taken.

Summary.

The formation of coloured condensation products from indole derivatives and certain aldehydes necessitates the use of condensing agents. The reaction takes place between the – NH group of the indole nucleus and the – CHO group of the aldehyde.

The evidence adduced in this paper is favourable to the Adamkiewicz reaction being primarily a formaldehyde reaction.

1. The formaldehyde condensation product of tryptophane gives the colour test directly with concentrated sulphuric acid, without the addition of an oxidising agent or of glyoxylic acid or of formaldehyde.

2. A trace of formaldehyde, without the addition of an oxidising agent, gives the colour reaction with indole derivatives.

3. When formaldehyde is used in testing for indole derivatives, the purple colour is often masked on account of further reactions taking place between the indole substance and the trioxymethylene formed from formaldehyde by the condensing power of the concentrated acid.

4. In Hopkins and Cole's modification of the test, glyoxylic acid reacts by virtue of its decomposition into formaldehyde, and for one of the two following reasons is the best reagent to use for the reaction:

(*a*) the formaldehyde is liberated in such small quantity that the formation of trioxymethylene does not take place to any appreciable extent,

(*b*) the formaldehyde so liberated is in the nascent state, and therefore more reactive.

5. In the case of tryptophane the colour test is often performed with formaldehyde in the presence of an oxidising agent. The part played by the latter is primarily to produce some oxidation product of tryptophane, e.g. indolealdehyde.

The reaction under these conditions is of a complex nature as, besides the

effects described above, (2) and (3), it involves the formation of coloured substances by the action of the concentrated acid on:

(*a*) indolealdehyde and tryptophane.

(*b*) indolealdehyde in the presence of an oxidising agent.

(*c*) indolealdehyde and tryptophane in the presence of an oxidising agent.

(*d*) indolealdehyde and tryptophane in the presence of formaldehyde.

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XII. THE RÔLE OF GLYCOGEN, LECITHIDES, AND FATS IN THE REPRODUCTIVE ORGANS OF ECHINODERMS.

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(*Received November 30th, 1912.*)

In the course of a research upon the protamines and histones of the spermatozoa of *Echinus esculentus* in which the reproductive organs (gonads) were extracted with hot alcohol, we were struck by the enormous amount of fatty matter removed by the hot alcohol. This separated out in drops and formed a thick layer at the bottom as the alcohol cooled. This appeared to us an unusual result to obtain from a male gland possessing only a reproductive function. It was still more surprising to find the aqueous extract made later on, in the process of extraction of the protamines, heavily laden with glycogen as shown by a marked pearly translucence, and confirmed by a marked iodine reaction even after manifold dilution. In both of these respects, the extracts of the gonads might well have been those from a liver or hepato-pancreas in a vertebrate or higher invertebrate, so rich were they in the usual products of metabolism. In the echinodermata, the gonads form by far the preponderating part of the soft tissues of the animal, and contain at least three-fourths of the organic matter of the organism. Also there exists in these animals no structure resembling a liver, or other metabolic gland.

It was accordingly determined to make analyses of the fatty constituents and glycogen of the gonads to elucidate their functions in this respect. For this purpose experiments were instituted to observe the effects of seasonal variation in April and in August—the period of sexual activity being in April; to keep the animals in batches for some weeks in the fed and unfed conditions respectively; and to test whether the gonads contained any diastatic enzymes.

The exceedingly slow metabolism of these organisms during even a lengthy period in which they were unfed, precluded us however from observing whether the reserve stores of fat and glycogen of the gonads could

be utilized for the purposes of the general metabolism of the animal as a whole. As is shown in another research [Moore, Edie, Whitley and Dakin, 1912, p. 287] upon the metabolism of marine organisms, the amount of organic matter required in twenty-four hours by an average-sized echinus is only 4·2 milligrams, and as the gonads contain about five grams of dry organic matter it is impossible to say whether these are acting as reservoirs for the minimal demands of the organism during deprivation of food even for a month or more. For example, 4·2 milligrams of organic matter oxidized daily, is equivalent to 30 milligrams of moist weight, and for one month, or 30 days, this would amount to a loss in weight of only 0·9 g. This much, however, is certain, that the gonads are very retentive of their stored-up food supplies, such as fats and glycogen, for after even three weeks' complete deprivation of food, the gonads are still very rich in both oils and glycogen, and even quantitative determinations show no constant difference between the fed and the unfed organisms. The unfed specimens are alive and well at the end of the period and commence at once to feed greedily when offered suitable food such as ordinary laminaria.

It is not to be supposed that the metabolism of the echinus is so excessively slow under normal conditions; this is only a dormant condition which can be assumed when there is necessity for it. When the organisms are first placed in captivity without food, there is an enormous output of faeces for the first two days, afterwards the water in the tank is practically free for the rest of the period. The animals also develop an appetite, which was shown by an attack upon the wooden bottoms of the tanks in which the unfed echini were stored. The unfed animals supported themselves in the stream of running water by the tube-feet just like the fed ones and appeared quite active and well in every respect.

The echini fed in the laboratory upon laminaria and other sea-weeds, ate quite vigorously and produced a large output of faeces daily, but at the end of the period, the extracts from the two sets of gonads, of fed and unfed, were very much alike in storage content. There may have been, however, an increase in bulk of the gonads, of those which were fed, for the size of the gonads varies considerably and they cannot be weighed at the beginning and end of the experiment.

Another important point to remember is that there is no trace whatever to be detected of a diastatic enzyme.

A gonad rich in glycogen may be left for some hours either at air temperature, or heated in a bath to 35°, for the same time, and at the end not a trace of sugar is obtainable.

It may even be left for a period of two days with the same result. But, extraction at the end shows the glycogen to be present in large amount, and on boiling with dilute acid a copious reduction with Fehling's solution is at once obtained.

The diminished metabolism during fasting, compared with the rapid uptake of food while fed, the persistence of fat and glycogen after long starvation, and the absence of a diastatic enzyme, when all considered together, and in conjunction with the relatively huge size of the reproductive organs, indicate that the stored fat and glycogen are intended for the internal metabolism of the gonads themselves and to subserve the enormously developed reproductive functions.

The main bulk of the metabolized food would also appear to be devoted to the nourishment and upbuilding of the reproductive organs. These organs appear during the resting season to build up rich nutritive material and then discharge this during the active season, when a diastatic ferment may perhaps be temporarily developed.

It is remarkable that a few months after the active season, as in August (the period of activity having been the end of March and earlier part of April), the gonads are as large and full as before activity, and richly charged with oils and glycogen. While being fed the uptake of material must be many times the amount required for locomotion and other physiological purposes in the unfed condition, as shown by the amount of oxygen used daily by the unfed animal. If it may be assumed that the unfed animal requires about as much energy for these purposes as the fed animal, as would seem to be shown by the activity of the two being similar, then it may be concluded that at least nine-tenths of the energy of the food taken up by the echinus is conserved for reproductive purposes.

In regard to the carbohydrate and fatty metabolism, there is no noticeable difference between the male and female gonads, both are rich in oils and in glycogen.

A considerable amount of lecithides was in all cases found admixed with the fats. The iodine value for both lecithides and oils was high, showing a marked degree of lack of saturation. In this respect the fats of the echinus resemble the fish-oils, or fish liver-oils.

Thus, for the oils, iodine values varying from 130 to 190 were obtained, and for the lecithides, values of 70 to 120. These high iodine values suggest that these fatty constituents may possess therapeutic values similar to those of cod-liver oil in wasting diseases.

Methods of Extraction and Analysis.

In the earlier experiments carried out at Easter 1911, only the fats, oils and lecithides as extracted by thorough treatment (*a*) with cold and (*b*) with hot alcohol afterwards, were obtained and analysed.

The work was resumed in August and September 1911, and Easter 1912, and then two methods chiefly were employed. In the first method the glycogen only was estimated, by boiling out the gonads twice with water acidulated with acetic acid, after which a third decoction showed no opalescence and gave very little reaction with iodine. The two extracts were added together, precipitated by the double volume of alcohol, washed with 70 per cent. alcohol by decantation, and preserved in this for some months, after which the precipitated impure glycogen was digested with strong caustic potash, as in Pflüger's method described in the concluding part of the second method below, followed by precipitation with alcohol, washing with alcohol, drying and weighing. In several cases, the amount of glycogen present was finally determined by hydrolysis to glucose, reduction by excess of Fehling's solution, and gravimetric determination of the CuO. When this was done it was found that the product of the Pflüger's method was never pure glycogen, but varied from 63 to 93 per cent. pure.

Both results are given alongside each other in the tables below for comparison. The nature of the impurity was not determined, but it indicates that this method of determining glycogen should be used with caution, and always followed by a hydrolysis and sugar determination.

Second Method. In this, the final method used, which was found by far the most convenient, oils, lecithides, and glycogen were all determined in the same specimen. At the end of the preliminary treatment of the organisms at the Biological Station, the shells were broken open by chipping round in a sectional plane midway between mouth and anus, and the sex determined by examining a smear from a gonad with a low power of the microscope.

The echini were thus sorted into two heaps, the five gonads were removed from each echinus, and two heaps made of male and female gonads respectively. The weight of each quantity in the fresh condition was taken, and they were then broken up and placed in about three times their volume of absolute alcohol. In this condition they were transported to Liverpool and after some months were then extracted for oils and lecithides, and for glycogen respectively by the following method.

The alcohol was decanted and filtered off, without previous heating. A

fresh quantity of absolute alcohol was then added to the tissue which was previously broken up as finely as possible, and mechanical agitation in a bottle on a shaker was used for some hours. This second quantity of alcohol was filtered off as before, and when mixed with the first, the mixture formed the "cold alcoholic extract."

The "hot alcoholic extract" was obtained similarly by two extractions with boiling alcohol, decantation and filtration.

Each alcoholic extract was subsequently treated in precisely the same fashion for determination (*a*) of salts and extractives, (*b*) lecithides, and (*c*) fats and oils, as follows:

The alcoholic extract was evaporated till all alcohol had been removed, and the total weight was taken. The residue was exhausted by repeated portions of methylated ether, which portions were all united. The residue from the ether consisted of salts, creatine, etc., and this was weighed separately. The ethereal extract was evaporated down cautiously to a thin syrupy consistency, acetone in excess was then added to precipitate the "lecithide" fraction, this was dried and weighed and constants determined. The portion soluble in acetone gave the product described under the heading oils and fats. It is, of course, only claimed that this is a rough separation. The labour of a complete separation in so many experiments would have been inordinately great, and the figures for the constants in the two respective columns (1) lecithides, and (2) oils and fats, show that these two fractions represent a fairly complete separation of the lecithides from the oils. The weight, saponification value, and iodine value, were determined in each case.

The dried tissue after removal of fats, etc., as above described, was weighed and then digested for three hours on a steam bath with 40 per cent. solution of caustic potash, which, as discovered by Pflüger, leaves the glycogen intact while it decomposes proteins. After cooling, twice the volume of absolute alcohol was added, so precipitating the glycogen. The glycogen was filtered off through a Gooch crucible provided with a filter of finest woven silk bolting such as is used for flour sifting. This was found to be much preferable to glass-wool, or asbestos, and may be recommended as a filtering medium for alkaline fluids. The precipitate is then washed with a 15 per cent. solution of caustic potash in a 66 per cent. solution of alcohol, as recommended by Pflüger. Next it is washed through into solution in a fresh receiver with hot water. The aqueous solution is again filtered, reduced in volume by evaporation if necessary, and finally precipitated by twice its volume of absolute alcohol, filtered, washed with two-thirds alcohol, dried and weighed. Finally, the content of glycogen in the product is determined

TABLE I.

Experiments of Easter 1911. *Determinations of Lecithides and of Fats and Oils in Ripe Ova and Spermatozoa of* Echinus esculentus.

Material and extractive	Total weight of extract	Salts, creatine, etc. extractives	Lecithide values			Fat and Oil values			Dry weight of residue after complete alcoholic extraction
			Total weight	Saponification value	Iodine value	Total weight	Saponification value	Iodine value	
			OVA.						
Exp. I. Ripe Ova, 580 g. moist weight:									grams
Cold alcohol	8·56	2·90	2·10	259	73·4	2·85	205	166	27·95
Percentages on moist weight ...	1·48	0·50	0·36	—	—	0·49	—	—	
Hot alcohol	11·44	0·35	2·34	207	76·0	8·13	192	136	
Percentage	1·97	0·06	0·40	—	—	1·40	—	—	
Exp. II. Ripe Ova, 332 g. moist weight:									
Cold alcohol	7·10	3·50	1·10	207	84·0	2·15	188	133	12·05
Percentage	2·14	1·06	0·33	—	—	0·65	—	—	
Hot alcohol	8·10	1·20	1·70	229	82·0	4·23	195	145	
Percentage	2·44	0·36	0·51	—	—	1·30	—	—	
			SPERMATOZOA.						
Exp. III. Ripe Spermatozoa, 700 g. moist:									
Cold alcohol	9·04	1·04	2·60	176	86·1	4·61	201	140	21·7
Percentage	1·29	0·15	0·37	—	—	0·66	—	—	
Hot alcohol	10·62	0·72	0·50	180	77·0	9·08	172	131	
Percentage	1·52	0·10	0·07	—	—	1·30	—	—	
Exp. IV. Ripe Spermatozoa, 235 g. moist:									
Cold alcohol	3·10	0·48	0·65	186	90·7	1·40	200	166	(Not taken)
Percentage	1·31	0·20	0·28	—	—	0·60	—	—	
Hot alcohol	7·67	0·75	1·80	190	81·0	3·33	197	153	
Percentage	1·28	0·32	0·77	—	—	1·43	—	—	

by hydrolysis, and gravimetric determination by precipitation in excess of Fehling's solution.

Experiments of Summer 1911 (*August and September*). The first four experiments to be described in this series were in regard to content of glycogen only, and the effects of lack of food upon the glycogen content of the gonads. The gonads in all these experiments are taken at an inactive sexual period. The ova in the females at this time of year are unripe and small, and formed eggs are small in number relatively to other tissue; the spermatozoa in the males are alive and moving, but small.

Thirty echini of medium and large size only were taken from Port Erin Breakwater at low tide on August 22, all were kept in a large tank filled with running sea-water until August 24 (about 48 hours), not being fed in the interval.

Twenty of the echini were then opened and the gonads examined for sex, twelve were found to be females and eight were males. The female and male gonads separately were at once treated for glycogen separation as will presently be described in Expts. V and VI. The remaining ten echini were kept in the tank in fresh running sea-water without any food until Sept. 11, that is for a period of twenty days in all; they were alive and well at the end of that period, and were then opened, when gonads, and all the other organs, presented quite a healthy and well-fed appearance: the intestines were of course empty. On examination of these for sex, four were found to be females and six were males. The two masses of female and male gonads were then at once treated for extraction of glycogen in the fresh condition with the results shown in Expts. VII and VIII.

Experiment V. The gonads from the twelve females described above weighed 440 g. in the moist condition or an average of 36·6 g. per animal. As subsequent determination of dry weight of a given weight of moist gonad gave a solid content of 17·2 per cent., this weight represents 75·7 g. of dry solid. This dry solid has salts present in the proportion of sea-water in invertebrates so that the amount of dry organic matter is about 62·5 g.

The material was mashed up into a soft pulp, and the proteins coagulated by dropping the pulped mass into 400 c.c. of boiling one per cent. acetic acid in distilled water, and boiling for five minutes. The fluid was then filtered off through fine silk gauze and pressed out. The residue of tissue was pounded up and extracted with 500 c.c. of distilled water in two portions. The last filtrate was almost clear of glycogen, and gave only a trace of reaction with iodine. The first filtrate in dilute acetic acid was a strongly opalescent solution but without obvious suspension. This was again filtered through

two folds of fine silk, and a small portion of the filtrate which was still strongly opalescent gave a strong port-wine colour with iodine, and on boiling for five minutes with 3 per cent. hydrochloric acid gave a copious reduction with Fehling's solution.

The main volume of the acetic acid solution and the washings were united and precipitated with twice the volume of absolute alcohol giving an abundant white precipitate settling easily. This was washed, after decantation, with more two-thirds alcohol, and carried under alcohol to Liverpool, where it was later passed through the Pflüger process for purification of the glycogen and analysed. The weight of glycogen obtained was 7·44 g., but hydrolysis and a gravimetric Fehling determination showed this to be only 79 per cent. pure glycogen giving 1·33 per cent. glycogen on the moist weight, or the glycogen in the gonad is 9·36 per cent. of the total organic matter.

Experiment VI. The eight sets of male gonads weighed 258 g., thus averaging 32·25 g. per animal. Of this quantity, 200 g. were taken for glycogen extraction, corresponding to 28·2 g. of dry organic matter. The tissue was put through exactly the same process as used for the female glands, and gave the same appearances and the same qualitative tests. The filtration of the dilute acetic acid solution was more difficult, probably on account of the presence of the minute spermatozoa, but obviously there was much glycogen present, as shown by fine opalescence and iodine test. After filtration through fine silk it was precipitated by two volumes of alcohol as before, and carried off for analysis. Any spermatozoa present in the original acetic acid precipitate would be destroyed by the subsequent boiling with strong alkali in the Pflüger process. The amount of glycogen obtained was 3·02 g., and this was found on hydrolysis to be 81·4 per cent. pure, corresponding to 1·51 per cent. of crude and 1·23 per cent. of pure glycogen in the moist gonad, or to 8·66 per cent. of the total organic matter.

It is thus seen that the percentage of glycogen is much the same in the male and female gonads, corresponding to 9·36 per cent. in the female and 8·66 per cent. in the male of the dry organic matter.

Experiment VII. The four sets of gonads from the females which had been kept unfed in the tank for 20 days weighed 122 g., or an average of 30·5 g. per echinus, while Expt. V shows that the twelve sets of gonads of females killed without previous abstention from food weighed 440 g., an average of 36·6 g. The difference is not great and is probably quite fortuitous, as the content of glycogen indicates no effects of the twenty-days' fast.

The process of extraction was exactly the same as in Expts. V and VI,

and yielded similar qualitative results. The yield of crude glycogen was 2·09 g. but this, for some unknown reason, showed only 62 per cent. of glycogen on hydrolysis, corresponding to 1·06 per cent. of the moist gonad, or 7·47 per cent. of the total organic matter. This is a somewhat smaller figure but the decrease is too small to mean any real demand on the gonads for reserve food by the rest of the organism during the period of inanition, also they are still very rich in glycogen. Moreover, the result is the reverse in the experiment with the males now to be described.

Experiment VIII. The six sets of gonads from males kept without food in the tank of running sea-water for 20 days weighed 237 g., thus averaging 39·5 g. per animal. These were extracted with dilute acetic acid and precipitated with alcohol at Port Erin on similar lines to the previous experiments, and the precipitated crude glycogen was afterwards worked up by Pflüger's method at Liverpool, and controlled by hydrolysis and estimation of glucose. There were obtained 4·45 g. of glycogen, shown by hydrolysis to be 82·8 per cent. pure; this works out at 1·54 per cent. of pure glycogen in the moist gonad, or 10·84 per cent. of the organic matter. It is here seen that the glycogen in the unfed males is somewhat higher than in the fed males of Expt. VI; but as the females are in the reverse direction by about a similar amount, the proper conclusion is that the four experiments demonstrate no appreciable disappearance of glycogen from the resting gonads of male or female as a result of a fast of twenty days. All four experiments concordantly show that the reproductive organs of *Echinus esculentus* are very rich in glycogen.

A second catch of 60 well-grown individuals was made from Port Erin Breakwater on August 25 at low water with which the remaining experiments of this series were carried out. All these were left over-night in a large tank through which there ran an abundant supply of fresh sea-water. Next morning a large amount of faecal matter obviously chiefly of vegetable origin was found on the bottom of the tank. On the morning of the day after capture (Aug. 26), 15 individuals taken indiscriminately were opened, examined for sex, and the gonads assembled in two lots, one male, the other female. Out of these 15 specimens, six were females, eight males, and one was rejected as being in an abnormal condition. The six female sets of gonads weighed 154 g., or 26·6 g. per individual; the eight male sets weighed 235 g., or 29·4 g. per individual. The mass of gonads in each case was sampled out into three portions which were used as follows: (1) was at once placed directly in alcohol, for determination later of oils and fats, lecithides, and glycogen, (2) was at once extracted with dilute acetic acid and

precipitated with alcohol, etc. for glycogen determination, as in the preceding four experiments, and (3) a smaller quantity was dried on a bath for determination of total solid matter in the fresh tissue of the gonad.

Out of the 154 g. of female gonad, 74·5 g. were placed in alcohol (see Expt. IX b), 61·2 g. were extracted fresh with dilute acetic acid (see Expt. IX a), and 14·26 g. were taken for determination of total solids, which were found to amount to 17·2 per cent., as the glands contain approximately 3 per cent. of inorganic salts, on the basis that their fluids are isotonic with sea-water this leaves 14·2 per cent. of dry organic matter in the female gonad.

Similarly the eight male gonads weighing 235 g. were divided into 75·1 g. placed directly in absolute alcohol (see Expt. X b), 134·4 g. extracted fresh with dilute acetic acid (see Expt. X a), and 12·75 g. taken for determination of total solids. The percentage of total solids found was 16·8, and after deduction for inorganic constituents this leaves 13·8 per cent. of dry organic matter in the tissue of the male gonad. This lies very close to the figure for the female organ given above, and the values of 17 per cent. for total solids, and 14 per cent. for organic solids, may be taken as an average.

The remaining 45 individuals of the second catch (Aug. 25) were used, as follows, for experimentation in the laboratory. They were divided into three lots of 15 individuals each, the sizes being arranged as far as possible so as to give no advantage to any one batch[1]. The first batch were left unfed in the large tank (see Expts. XI and XII). The second and third batches were removed in order to have plenty of room and fresh running sea-water, to a series of smaller tanks forming part of a fish-hatching apparatus not at the time in use. The thirty echini were distributed over ten of these tanks, three in each tank. Fifteen of these echini were fed on fresh laminaria gathered on the shore (see Expts. XII and XIII) and they ate a surprisingly large amount of it daily. The other fifteen, forming the third batch, received no food of any kind during the period of the experiment (see Expts. XIV and XV). As some index of the rate of feeding of the echini supplied with laminaria there may be mentioned the amount of faeces found in the tanks.

The faecal masses were washed into a graduated 100 c.c. measure, allowed to sediment, and the volume of the sediment measured. The volumes found were as below. At this time in the experiment a few of the echini had perished, as noted below:

Tank No. I. Two echini, 45 c.c. of faecal sediment in three days.

[1] As a matter of fact it frequently happens that heavier gonads are obtained from a medium-sized than from a large echinus, so that this precaution is scarcely required so long as no individuals below a certain size are taken in the first instance.

Tank No. II. Two echini, 35 c.c. of faecal sediment in three days.

Tank No. IV. Three echini, 100 c.c. of faecal sediment in three days.

The amount of laminaria eaten was not actually weighed, but it was quite a surprising amount for organisms of so slow a metabolism, and was evidently chiefly being used for storage in the gonads. The unfed batches of these echini produced no faeces, after the first two days or thereabouts, and at the end of the experiment it was noticed that in the tanks of the fish-hatching apparatus, which were made of planed wood, in each case four or five bitten out spots showed where the animals had attacked the wood of the tank floor and scooped it out quite distinctly; no similar attack had been made on the tanks containing those fed on laminaria. The experiment lasted from August 25 till Sept. 13, a period of nineteen days. At the end the gonads were removed, examined for sex, and placed at once in absolute alcohol, from which they were taken and analysed some months later in Liverpool.

Early in this experiment a certain number of the echini in each of these three batches succumbed. This result was in no wise due to lack of food, for as great a percentage died in the fed batch as in the corresponding unfed batch. Such mishaps often occur soon after the echini have been taken, for some unknown reason, and then the survivors keep in quite good condition. All the animals alive at the end were active and appeared quite normal and healthy. Out of the fifteen kept in the large tank, nine survived, of which six were males and three were females; of the fifteen fed daily in the fish-hatching tanks, eleven survived, seven females and four males; of the fifteen kept unfed in fish-hatching tanks, eleven also survived, six males and five females.

Experiment IX a. Amount of glycogen in freshly extracted female gonads. This experiment was carried out with a portion, 61·2 g., of the gonads of females of Catch II, freshly extracted with dilute acetic acid. Details as before, amount of glycogen 1·14 g., found on hydrolysis 87·7 per cent. pure = 1·63 per cent. of pure glycogen in fresh gland, or 11·64 per cent. of the dry organic matter.

Experiment IX b. Determinations of (*a*) lecithides, (*b*) fats and oils, and (*c*) glycogen in a portion, 74·55 g., of female gonads of Catch II, placed at once in alcohol. Hot and cold alcoholic extracts were taken and analysed as described, and the glycogen determined in the residue. In percentages of the moist tissue, the following were the results obtained: glycogen, 1·53 per cent.; lecithides, cold alcohol 0·41, hot alcohol 0·25, total = 0·66 per cent.; constants (cold alcohol), sapon. value 210, iodine value 78·0; (hot alcohol), iodine value 91·1. Fats and oils, cold alcohol 0·4, hot alcohol 1·46, total =

1·86; constants (cold alcohol), sapon. value 208, iodine value 180; (hot alcohol), sapon. value 197, iodine value 174.

Percentages expressed on dry organic matter: glycogen, 10·9; lecithides, 4·6; fats and oils, 13·1.

Experiment X a. This was carried out upon a portion, 134·4 g., of the male gonads of Catch II, freshly extracted with dilute acetic acid, etc. Amount of glycogen obtained, 2·66 g. of 81·15 per cent. purity = 1·60 per cent. of glycogen in fresh gland, or 11·3 per cent. of the dry organic matter.

Experiment X b. Determinations of (*a*) lecithides, (*b*) fats and oils, and (*c*) glycogen in a portion, 75·1 g., of male gonads of Catch II, placed at once in alcohol. Details as in Expt. IX b. Results obtained in percentages of moist tissue: glycogen, 1·70; lecithides, cold alcohol, 1·01, hot alcohol 0·26, total = 1·27; constants of lecithides, (cold alcohol), sapon. value 240, iodine value 89, (hot alcohol), sapon. value 197, iodine value 98·2; fats and oils, cold alcohol 0·54, hot alcohol 1·80, total = 2·34; constants of fats and oils, (cold alcohol), sapon. value 206, iodine value 160, (hot alcohol), sapon. value 202, iodine value 181.

Percentages expressed on dry organic matter: glycogen, 11·90; lecithides, 8·89; fats and oils, 16·38.

Attention may again be drawn to the highly unsaturated nature of the fats and oils as shown by the high iodine values, in both male and female glands, and to the high percentages of reserve storage materials throughout. There was also a large amount of organic matter soluble in 66 per cent. alcohol, and a relatively small amount of insoluble protein and tissue debris, but figures for these are not given, as analyses of the portions were not made. The results were similar in this respect to Table II in which the total amount of salts and alcohol soluble extractives are shown in a similar set of experiments.

Experiments XI to XVI. The whole of the glands in each of these experiments were placed at once, after conversion into a soft pulp, in twice their volume of alcohol. Through a misunderstanding they were used for the glycogen determinations after having been extracted with cold alcohol only, so that the figures given below do not represent total amounts of lecithides, or fats and oils. They are comparable amongst each other, however, and show that there is no appreciable diminution of fatty substances as a result of the nineteen-days fast.

Experiments XI and XII refer to the unfed echini in the large tanks; there were three females, weight of gonads 73 g., average 24 g., and six males, weight of gonads 152 g., average, 25 g. Experiments XIII and XIV refer

TABLE II.

Portion of Experiments of Summer 1911. *Determinations of Lecithides, Fats and Oils, and Glycogen in Unripe Ova and Spermatozoa of* Echinus esculentus. *The experiments show results, in animals kept, fed and unfed, during a period of* 19 *days in laboratory* (*Port Erin I.O.M.*).

Nature of material extracted with cold alcohol	Total weight of extract	Salts, creatine, nitrogenous bases, etc.	Lecithide values: Total weight	Lecithide values: Saponification value	Lecithide values: Iodine value	Fat and Oil values: Total weight	Fat and Oil values: Saponification value	Fat and Oil values: Iodine value	Glycogen
	Exps. XI and XII. **Unfed** animals together in large tank.								
Exp. XI. Ova unfed:									
Weights in 73 g.	11·1	8·90	1·16	181	100	0·74	203	186	0·56
Percentages of moist tissue ...	15·2	12·19	1·59	—	—	1·01	—	—	0·77
Percentages of dry organic matter	—	—	11·13	—	—	7·07	—	—	5·39
Exp. XII. Spermatozoa unfed:									
Weights in 152 g.	13·55	10·25	1·66	225	125	1·5	230	170	2·41
Percentages of moist tissue ...	8·91	6·74	1·09	—	—	1·0	—	—	1·59
Percentages of dry organic matter	—	—	7·63	—	—	7·0	—	—	11·13
	Exps. XIII and XIV. **Fed** animals in fish-hatching tanks.								
Exp. XIII. Ova fed:									
Weights in 204 g.	14·1	11·20	1·88	187	112	0·85	220	180	2·44
Percentages of moist tissue ...	6·9	5·49	0·92	—	—	0·41	—	—	1·20
Percentages of dry organic matter	—	—	6·44	—	—	2·87	—	—	8·40
Exp. XIV. Spermatozoa fed:									
Weights in 118 g.	9·30	6·90	1·37	212	118	0·95	209	191	1·20
Percentages of moist tissue ...	7·90	5·85	1·16	—	—	0·80	—	—	1·02
Percentages of dry organic matter	—	—	8·12	—	—	5·60	—	—	7·14
	Exps. XV and XVI. **Unfed** animals in fish-hatching tanks.								
Exp. XV. Ova unfed:									
Weights in 173 g.	13·5	9·85	2·11	189	108	1·20	227	166	0·51
Percentages of moist tissue ...	7·8	5·69	1·22	—	—	0·69	—	—	0·30
Percentages of dry organic matter	—	—	8·54	—	—	4·83	—	—	2·10
Exp. XVI. Spermatozoa unfed:									
Weights in 178 g.	14·01	10·5	1·20	181	101	1·95	189	152	2·00
Percentages of moist tissue ...	7·88	5·9	0·67	—	—	1·10	—	—	1·13
Percentages of dry organic matter	—	—	4·69	—	—	7·70	—	—	7·91

to echini fed during these 19 days with laminaria, but kept otherwise under strictly similar conditions to those in the two subsequent experiments (Expts. XV and XVI); there were seven females, weight of gonads 204 g., average 29 g., and four males, weight of gonads 118 g., average 29 g. Experiments XV and XVI refer to echini unfed, but otherwise similar to those in Expts. XIII and XIV; there were five females, weight of gonads 173 g., average 34 g., and six males, weight of gonads 178 g., average 29 g. The average total weight of gonads is less in the case of the unfed echini in the large tank, but in those unfed, alongside the fed, in the fish-hatching apparatus no such effect is discernible. In fact the gonads of the unfed females are appreciably heavier than those of the fed females, while the males are practically of equal weight in the two series. The only conclusion valid is that even a nineteen-days period of removal of supplies is ineffectual to cause any real starvation, or appreciably use up reserves.

It would certainly have been expected from the amount of laminaria used by the echini that some increase of weight might have taken place in the fed animals, but it is possible that only a small amount of the nutritive organic matter of the laminaria was actually utilised by the echini. Certainly the faeces still contained a large amount of nutrition for they were swarming with copepoda when examined microscopically. These no doubt came in with the water supply and remained and accumulated upon the faeces of the echini, which formed their food supply.

The analytical results of these six experiments are shown in Table II. The figures obtained in these experiments show no clear evidence of diminution of reserves in the glands as a result of stopping the feeding for nineteen days, for there is still abundance of glycogen and fatty materials.

Experiments of Easter 1912. The echini used in these experiments were taken a week or two after the active sexual period which appears to have been somewhat early in this particular Spring. At least it was difficult to obtain any ripe eggs for fertilisation about the period of this experiment. The catch was made on April 10 and was divided into two lots, the animals of one of which were kept unfed from April 12 till April 21, while those of the other lot were fed on laminaria. The animals were killed at the end by opening the shells, examined for sex, and separated into four heaps, male and female, fed and unfed, respectively. These were immediately fixed by placing in two volumes of alcohol and taken to Liverpool for the analyses, which were conducted as described above; the results are given in Table III. It will be observed that the results do not differ materially from the others. Only percentage figures and the constants are given in the table.

TABLE III.

Organs extracted	Lecithides: Percentage of dry organic matter	Lecithides: Saponification value	Lecithides: Iodine value	Oils: Percentage of dry organic matter	Oils: Saponification value	Oils: Iodine value	Glycogen: Percentage of dry organic matter
Exp. XV. Sperm. of fed males, wt. 205 g.							
Cold alcohol	5·1	159·6	86·0	4·1	164·7	150·1	—
Hot alcohol	7·2	176·1	90·1	5·0	122·3	133·4	—
Total	12·3	—	—	9·1	—	—	7·6
Exp. XVI. Ova of fed females, wt. 171 g.							
Cold alcohol ...	2·87	168·1	82·8	3·15	178	134·7	—
Hot alcohol ...	3·92	172·8	89·7	3·92	185·1	129·5	—
Total	6·79	—	—	7·07	—	—	6·2
Exp. XVII. Sperm. of unfed males, wt. 62 g.							
Cold alcohol ...	9·10	163·4	94·5	10·43	158	148·6	—
Hot alcohol ...	1·12	174·3	101·0	1·12	112·1	160·6	—
Total	10·22	—	—	11·55	—	—	11·1
Exp. XVIII. Ova of unfed females, wt. 160 g.							
Cold alcohol ...	4·08	164·2	88·6	6·08	168·1	142·1	—
Hot alcohol ...	3·76	171·7	96·3	11·68	174·2	118·4	—
Total	7·84	—	—	17·76	—	—	9·1

Conclusions.

1. Both male and female reproductive glands in echinoderms contain large amounts of reserve metabolic products such as glycogen, fats and lecithides.

2. These reserves are only slowly used up, if at all, when the animal is deprived of food.

3. In a reproductive gland richly stored with glycogen, no sugar formation occurs on keeping after death, even in a period of two days.

4. The amount of food consumed is much greater than that required to cover the daily metabolic wants of the animal, and is largely stored in the reproductive glands during the resting period, but it has not yet been possible to trace the conversion of this at the active reproductive season.

5. The fatty constituents of the reproductive organs of the echinoderm are highly unsaturated, and resemble in this respect liver oils.

We desire to acknowledge much valuable assistance from Messrs. W. H. Evans and T. A. Webster in connection with the experimental work.

REFERENCE.

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XIII. THE BASIC AND ACIDIC PROTEINS OF THE SPERM OF *ECHINUS ESCULENTUS*. DIRECT MEASUREMENTS OF THE OSMOTIC PRESSURE OF A PROTAMINE OR HISTONE.

By BENJAMIN MOORE, EDWARD WHITLEY and ARTHUR WEBSTER.

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(*Received December 20th, 1912.*)

So far as we are aware no one has hitherto made a determination of the molecular weight in colloidal solution of any member of those interesting classes of proteins with marked basic properties known as the protamines and histones. These substances have been set down by workers upon them such as Miescher and Kossel as the simplest steps in natural protein synthesis which furnish a clue as to the constitution of the others, and it is rather remarkable that no attempts have been made to study their degree of complexity in solution. The present paper must only be regarded as a tentative experiment in this direction, to be made more complete when more material becomes available; for the chief difficulty lies in obtaining suitable material and working it up into a supply of pure protamine or histone. The substance which we have isolated from the ripe male gonads of *Echinus esculentus* appears to us to stand intermediately between a protamine and a histone, for it gives feebly the Millon's test as a reddish colouration on boiling, with no precipitate; and yet it possesses all the properties of a protamine. This substance dissolved as a sulphate in distilled water gave an osmotic pressure leading to a molecular weight in solution much less than that of an albumin or globulin, the figure being approximately 8780 when calculated to standard temperature and pressure.

It behaved as a true colloid in being perfectly indiffusible through parchment paper, and the osmotic pressure rose steadily to a maximum, and remained there at a constant level. Afterwards, the sulphate of the protein

was thrown out in apparently quite unaltered form by excess of alcohol, dried and weighed. The substance comes down quite sharply with excess of alcohol, as a white precipitate readily soluble again in distilled water. As this process was repeated several times before measurements were taken, it is quite certain that if there was any admixture it must have been that of very closely allied substances of this class.

The only other substance of this protein group from the echinodermata ever examined was a substance isolated from the sperm of *Arbacia* by Matthews [1897], and called by him *arbacin*. This substance is classed amongst the histones by Matthews chiefly because it gave a positive result with the Millon's test. The substance with which we were working, from a different species of echinus, in a first preparation gave a negative reply to the Millon's reagent, and in a second preparation gave a somewhat feeble red colouration but no precipitate, and in absence of more complete analysis we are consequently somewhat doubtful as to whether we ought to classify it as a protamine or histone, so for the present we prefer to leave the point open.

It may be of some interest to pause here to state that our original intention in isolating this substance at Port Erin in the Spring of 1911, just at the commencement of the active sexual period, was to observe whether protamines and histones acted as hormones to the earlier stages of cell-division in sexual reproduction.

The whole group of the protamines is intimately connected with the male reproductive organs, being found in these glands almost exclusively in fishes, and in greatest abundance just before the sperm cells are ripe for discharge. The histones are obtained from the ripe spermatozoa of fishes, but in addition extend up to the sperm cells of mammalia. Both protamines and histones are strongly basic bodies, showing a markedly alkaline reaction when in solution as free bases, and combining strongly with acids, such as sulphuric acid, in definite molecular proportions.

Now it was shown some years ago by Moore, Roaf, and Whitley [1905] that almost infinitesimal amounts of alkali added to sea-water increased the rapidity of cell-divisions in fertilized echinus eggs, and that a slightly greater amount of alkali hurried the process into marked abnormalities.

It was accordingly thought that small amounts of alkaline proteins, such as protamines or histones, present in the sperm, ought to have prominent functions in relationship to cell-division.

It was from this point of view that the protamine, or histone, from echinus sperm was prepared and its action tested (*a*) upon unfertilized ripe

eggs and (*b*) upon fertilized ripe eggs, alongside controls developed in sea-water only.

The results were uniformly negative: whether this may be due to our being unable to realize proper experimental conditions we do not know. It may well be, that protamine added as sulphate to sea-water is in some wise rendered inert and incapable of penetrating the ovum, while the same substance carried into the ovum as part of the sperm-nucleus may be most effectual. The occurrence of these peculiar proteins in the sexual organs would seem to indicate some relationship with a reproductive process, but we have quite failed to realize an experimental proof.

The only positive result obtained by treatment of the fertilized eggs was that an extract of the crude sperm powder, after treatment with alcohol as described below, added in small amount, such as 1 in 4000, to sea-water inhibited cell-division and induced irregularities. Sperm nuclein appeared to possess a like action, but the protamine sulphate was quite inert.

The substance was separated from the male gonads by the well-known process of Kossel. About sixty well-grown echini were captured upon the breakwater at low tide on April 14th, 1911. The gonads of each individual were examined microscopically for sex identification and a mass of about 700 g. of spermatic gonads was obtained. This material was broken up finely and taken out in a large volume of water acidulated with acetic acid which causes the sperm cells to agglutinate and separate off. This was collected, extracted once with cold alcohol, and then exhausted of all fats and lecithides by four extractions with hot alcohol, and one with ether. The ether took out practically nothing on account of the previous exhaustive treatment with hot alcohol. The dried residue from all this extraction, of 700 g. of moist gland, amounted to only 21·68 g. This dried material remaining to the end, contains the protamines or histones and the nucleins or nucleic acids in combination together as nucleoproteins. To obtain the basic proteins (protamines or histones) it is extracted, still following Kossel's instructions, with five times its volume of half per cent. sulphuric acid, shaking vigorously at each extraction for about 15 minutes, followed by separation through fine silk gauze and pressing out. About three extractions were found to exhaust all protamine or histone from the material. After subsequent complete filtration through filter paper, the sulphuric acid extracts were precipitated with three volumes of absolute alcohol, yielding a nearly pure white precipitate which after standing for 24 hours was decanted and filtered by a Buchner filter. The precipitate dissolved readily in distilled water, and was purified by dissolving and reprecipitating with alcohol four times. It was

then dried after washing thoroughly with ether and weighed. The weight of the precipitate from 700 g. of moist gland, or 21·68 g. of dried tissue residue after alcoholic and ethereal extraction, was 1·95 g.

The residue from the extraction of the tissue with dilute sulphuric acid is exceedingly rich in nucleins or nucleic acid. It was freed from acid and dried by extraction with alcohol and ether, and then left to extract over night in the cold with one per cent. solution of caustic soda. When treated with the dilute alkali it swells up into a jelly which is almost solid in consistency and cannot be filtered in this condition, but it aggregates somewhat on adding three times its volume of absolute alcohol. It was left over night in this condition, but next morning was still unfilterable through paper. It was cleared of grosser debris by filtration through the finest silk bolting so giving a colloidal suspension free from large particles. When this alkaline alcoholic suspension was made just acid by addition of normal hydrochloric acid, a white flocculent precipitate was obtained which settled rapidly. This filtered quite readily leaving a very fine almost white precipitate on the paper. This precipitate was washed with distilled water over night, and until the washings were practically neutral next morning. It contained no protamine or histone as was shown by extracting it with dilute sulphuric acid and none was obtained from it at any stage, showing that the 1·95 g. mentioned above represented practically the total amount in the glands. The above precipitate caused by the acid in the alkaline extract, representing nuclein or nucleic acids, was weighed and found to be 7·45 g. A second extraction of the tissue residue separated by the silk filtration, yielded by the same method of alkaline extraction, followed by alcohol and acid, about 1·12 g. of a similar nucleic product, so that the total amount of acidic material was $7{\cdot}45 + 1{\cdot}12 = 8{\cdot}57$ g. in combination with 1·95 g. of basic protein. The final residue after extraction with both acid and alkali was a substance which swelled up into a thick jelly in dilute alkali but did not dissolve in it, and shrank up when treated with dilute acid and was obviously an insoluble fibrinous form of protein. The weight after treating with alcohol, drying and weighing was 9·10 g. This material, calculating from the above weights, and the total weight of dried tissue extracted, is seen to form roughly half of the organic residue remaining after extraction with dilute acetic acid followed by alcohol and ether.

A second extraction of a different batch of male gonads made afterwards in Liverpool gave similar results.

A small amount of the protamine (or histone) sulphate was used at Port Erin for the investigation of effects upon cell-division, the remainder was

afterwards made up into a solution in distilled water containing approximately two per cent. of protamine sulphate. This was introduced against distilled water in the type of osmometer already described in previous papers [Moore and Roaf, 1907]. The dialysing membrane was parchment paper and complete equilibrium was established in three days' time as shown by the figures given below. At the end negative results were obtained on testing for protamine on the water-side. The protamine solution on the solution side of the membrane was quite sweet at the end of the experiment, and the protamine was precipitable from solution by alcohol as at the beginning. The solution had of course become somewhat diluted by expansion, and a determination of the amount of protamine sulphate by precipitation by excess of alcohol, filtering on to a Gooch crucible, drying by washing with alcohol and ether and weighing gave 1·65 per cent. of protamine sulphate. This gave a steady osmotic pressure of 34 millimetres of mercury at 15°, which works out at 19·3 millimetres for a one per cent. solution at 0°, and leads to a molecular weight in solution of the protamine solution of 8780.

The following is the protocol of the experiment:

Date	Time	Manometric reading		Temperature
1911. Nov. 21st	12 noon	388		15°
,, 22nd	9.30 a.m.	391		13·5°
,, 23rd	9.45 a.m.	396		14·0°
,, ,,	5.30 p.m.	397		16·5°
,, 24th	10.0 a.m.	401		15·0°
,, ,,	6.0 p.m.	403		16·0°
,, 25th	9.45 a.m.	406	Constant	14·5°
,, ,,	5.45 p.m.	406		15·5°
,, 26th	10.0 a.m.	406		14·5°
,, ,,	5.30 p.m.	406		15·5°
,, 27th	9.30 a.m.	406		15·0°
Opened. Zero Pressure		389		

Osmotic Pressure = 2 (406 − 389) = 34 millimetres of mercury. Percentage of protamine sulphate = 1·65.

As has clearly been shown by Moore and Roaf, and confirmed by Lillie and others, the molecular weight of colloids in solution varies with the solvent, amounts of neutral salts, and acidity or alkalinity. Such reagents affect the colloidal aggregation. Still it is of interest to compare the complexity of solution aggregation of this basic protein, with averages for other proteins, and colloids.

The following results have been obtained by various observers:

Colloid	Osmotic pressure for one per cent. of Colloid	Molecular weight in solution
Gelatin (Moore and Roaf)	9·2 mm.	18,500
Egg albumin (Moore and Parker) ...	13·1	13,000
Serum (,, ,,) ...	2·8	60,800
Alkalized Serum (,, ,,) ...	17·0	10,000
Sodium Oleate (,, ,,) ...	10·4	16,300
,, Palmitate (,, ,,) ...	10·0	17,000
,, Stearate (,, ,,) ...	9·4	18,000
Caseinogen (Moore, Roaf and Webster) ...	129·0	1,400
Haemoglobin (Hüfner and Gausser) ...	—	15,000 to 16,000
,, (Reid)	—	65,000
,, (Roaf) under varying conditions from ...	—	5,000 to 70,000
,, but under more natural conditions and in neutral solution about ...	—	14,000 to 18,000
Histone and Protamine (present communication)	19·3	8,780

The solution complexity accordingly is less than that of the coagulable proteins, but is considerably higher than that of the acidic protein caseinogen, which is by far the lowest of all proteins hitherto examined. The alkalized serum and the histone lie nearly at the same level.

It is interesting to compare the molecular weight in solution as given directly by osmotic pressure with the molecular weights assigned from analysis by Kossel and his co-workers, and with the molecular weights of the amino-acids (hexone bases) which the protamines yield on hydrolysis. The molecular weights given by Kossel for salmine and sturine lie between 800 and 900, so that even after allowance is made for the inorganic acid in the sulphate there must be eight or more such large molecular groups in the solution aggregate to yield 8,000 to 9,000 as found. The mean molecular weight of the amino-acids forming this class of protein may be taken as 150, and, making allowance for the sulphate in combination, this leads to the conclusion that there are about 40 molecules of such amino-acids united to form the complex, or colloidal solution aggregate, of the protamine, or histone, in solution.

At present, similar measurements of the molecular complexity in solution of nucleoproteins and nucleic acids are being carried out.

The expenses of this research have been defrayed by a grant from the Government Grant Committee of the Royal Society.

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XIV. THE FATTY ACIDS OF THE HUMAN BRAIN.

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(*Received December 17th, 1912.*)

In the following communication evidence is brought forward that the solid fatty acids derived from the lipoids of the human brain do not consist chiefly of palmitic and stearic acids as has been claimed by certain investigators. The observation of Thudichum [1901] that the stearic acid of the human brain melts not at 69° but at 52° has been confirmed, and by fractional precipitation an acid isomeric with stearic acid has been isolated.

I have been unable to find any unoxidised fatty acids containing more than eighteen carbon atoms in the molecule. It has also been shown that at least 25 % of the solid fatty acids of the human brain are hydroxy-acids of higher molecular weight than stearic acid. From the close resemblance in properties which the cerebral hydroxy-acids show to the fatty acids found by Darmstaedter and Lifschütz [1896] in lanoline, it may be of some interest to suggest that we have here another indication of the relationship of the nervous system to other tissues of epiblastic origin.

The cause of the high molecular weight of the brain fatty acids.

About two years ago I pointed out [1910] that the mean molecular weight of the solid fatty acids of the human brain, as determined by titration, was considerably higher than that of stearic acid, this fact being confirmed by analysis of the lead soaps. I was not at that time acquainted with Thierfelder's paper on cerebronic acid [1905]. This acid occurs with other hydroxy-acids of high molecular weight in the precipitate of potash soaps difficultly soluble in alcohol, which is obtained when the saponified mixture from the fatty acids is allowed to cool. Moreover although the potassium salts of these hydroxy-acids are sparingly soluble in alcohol, they remain dissolved to a considerable extent in the presence of the other soaps and

subsequently separate amongst the first fractions precipitated as barium or magnesium soaps. The existence of hydroxy-acids in the brain other than cerebronic acid, with the possible exception of hydroxystearic acid [Koch, 1902] has not previously been noted. It is to the presence of these hydroxy-acids, however, that the observed high molecular weights are to be attributed. In the case of certain fractions it was observed that the apparent molecular weight varied enormously when the acid was regained by decomposition of the lead, barium or even the sodium soap by hydrochloric acid, the acid so regained possessing a higher molecular weight than before. The same phenomenon was observed in another way. When certain fractions of solid fatty acids of high molecular weight were converted into lead soaps, and these lead soaps subsequently exhausted with hot benzene or toluene (a process which Fränkel [1909] used to purify the lead soaps of the brain fatty acids), a decomposition sets in with the formation of a white precipitate rich in lead, and a brown coloured solution, which contains not lead soap, nor free fatty acid, but a material practically devoid of acid properties. It seemed clear that an anhydride or lactone formation had taken place in both the cases mentioned. That this view is correct is confirmed by the following fact. If the fatty acids are regained from the potash soaps difficultly soluble in alcohol, taking no precaution to avoid the dehydrating effects of mineral acids, it is found that the acid mixture so obtained is sparingly soluble in alcohol even at the temperature of ebullition, and after repeated exhaustion with hot spirit a portion is left which when dissolved in a mixture of chloroform and alcohol is found by titration to be only faintly acid; but if the same material is saponified the soap will be found completely soluble in hot alcohol and the material regained by decomposition with acetic acid is a true acid substance neutralising a considerable proportion of alcoholic potash. It is to the presence of anhydride or lactone-like derivatives of the hydroxy-acids that the observed variations in mean molecular weights as determined by titrating with potash, are due.

The lactone formation under the conditions described occurs most markedly, not with cerebronic acid, but with the hydroxy-acid melting at 73·5°.

Preparation of the fatty acids from the brain substance.

The brain after removal of connective tissue and washing was heated with 20 % potash solution. After cooling, the solution was acidified with hydrochloric acid and the fatty layer filtered off. This was then saponified with 10 % alcoholic potash for 15–24 hours. The solution was evaporated to

dryness with previous admixture of sodium bicarbonate, and the powdered residue extracted with anhydrous ether till the cholesterol was completely removed. The details of this process are described elsewhere [Grey, 1912]. The fatty acids were now converted into lead soaps and treated with ether, which was done with the aid of the centrifuge. The lead soaps insoluble in ether yielded acids with an iodine value of 27·5 %. From 150 g. of total fatty acids were obtained 67 g. of acids forming lead soaps insoluble in ether. The mean molecular weight of the solid acids was 320 as determined by titration. The iodine absorption is due chiefly to admixture with a solid unsaturated acid described later. The fatty acids from the lead soaps insoluble in ether were now neutralised with potash. The precipitate which separated on cooling is represented by fraction No. 1 in the accompanying table, and consists chiefly of hydroxy-acids. The filtrate was subjected to fractional precipitation by magnesium acetate, and the fractions 2–5 obtained. The substance remaining in solution (fraction 6) consists chiefly of the unsaturated acid melting at 42° to the presence of which the iodine-absorbing power of the solid fatty acids is due, earlier fractions being practically devoid of iodine value. The large loss of material was due to the volatilisation of the acids by steam.

TABLE I. *Showing the separation of* 58 *g. of solid fatty acid from the human brain.*

No. of fraction	How obtained	Weight	Melting point	Mean mol. weight by potash titre
No. 1	From potash soap insoluble in cold alcohol	14·6	84 –84·5°	420
No. 2	From first precipitate of magnesium soap	6·8	68·5–69°	399
No. 3	2nd ditto	6·5	55·5–56°	324
No. 4	3rd ditto	5·4	57 –57·5°	303
No. 5	4th ditto	8·0	47·2–47·6°	251
No. 6	Residue in solution ...	6·0	40 –41°	—
		Loss 10·7		

The hydroxy-acids of the human brain.

These were obtained from fraction No. 1 (*vide* Table I) and represent the acids forming potash soaps sparingly soluble in alcohol. They also separate from fractions 2 and 3 during the further purification of these. The potash soap was brought into solution by boiling alcohol and partially precipitated by magnesium acetate. The hydroxy-acid mixture so obtained was further fractionated by cooling the hot alcoholic solution of the acids, by the repetition of which process a fraction was obtained almost

insoluble in alcohol even when hot. This acid when dry was a white powder, and melted at 100–101°. The acid is not cerebronic acid. The main portion of the hydroxy-acids consisted of two other substances which were separated by recrystallisation from acetone and glacial acetic acid. The portion most soluble in acetone melted at 73·5°. The same substance was also obtained from the earlier fractions of magnesium soaps obtained from the potassium soaps soluble in alcohol.

In the course of these investigations the possibility of the formation of hydroxy-acids was considered, but since the chief hydroxy-acids found were not dihydroxy-acids, but monohydroxy-acids, they could not have been artificial products. It is possible however that small quantities of dihydroxy-acids may have resulted from the oxidation of unsaturated acids present. Experiments done with coal gas yielded practically the same iodine value as when no special precautions were taken. Moreover the isolation of acids of great degree of unsaturation and in particular of one containing six double bonds, indicates that oxidation during manipulation does not play any considerable part.

Nature of the hydroxy-acids.

Three distinct hydroxy-acids have been separated, but small quantities of others seem also to be present. The three acids isolated melted at 73·5°, 91° and 100–101° respectively. The melting point of this last corresponds to that of cerebronic acid, the quantity obtained was small, but judging by the result of a single combustion analysis the substance is certainly not cerebronic acid. On the other hand the acid melting at 91° corresponds closely in composition with cerebronic acid as determined by many analyses. The melting point of this substance remained constant after many recrystallisations from acetone and glacial acetic acid. The analyses of these acids are set forth in the accompanying table.

TABLE II.

No.	Melting point	Weight taken	CO_2	H_2O	C %	H %	Empirical formula
1	100–101°	0·1296	0·3395	0·1376	71·44	11·80	$C_{17}H_{34}O_3$ or $C_{22}H_{44}O_4$
2 (*a*)	91·0°	0·1077	0·2960	0·1193	74·956	12·308	
(*b*)	,,	0·1034	0·2851	—	75·174	—	
(*c*)	,,	0·1967	—	0·2270	—	12·530	
(*d*)	,,	0·1327	0·3660	0·1500	75·221	12·544	
(*e*)	,,	0·2267	0·6267	0·2567	75·420	12·590	
				Mean	75·193	12·493	$C_{25}H_{50}O_3$
3	73·5°	0·1433	0·3804	0·1730	72·398	12·073	
	,,	0·1323	0·3526	—	72·614	—	
				Mean	72·506	12·073	$C_{20}H_{40}O_3$

The composition of Thierfelder's cerebronic acid ($C_{23}H_{50}O_3$) is C = 75·38 %: H = 12·56 with which substance No. 2 will be seen to be in good agreement.

Substance No. 1 corresponds to the formula $C_{17}H_{34}O_3$ or $C_{22}H_{44}O_4$. The latter formula would be preferable since according to its melting point and other physical properties it can hardly be a lower member of the cerebronic acid series. In this case the acid would be a dihydroxy-acid and might have resulted by the oxidation of a pre-existing unsaturated acid of the formula $C_{17}H_{32}O_2$. Such an unsaturated acid has not yet been described as occurring in brain lipoids.

The acid melting at 73·5° corresponds approximately to the formula $C_{20}H_{40}O_3$ but it cannot be regarded as having been yet satisfactorily examined. It is characterised by the readiness with which it may be transformed into a non-acid lactone-like substance, as already explained.

The unsaturated fatty acids of the human brain.

Since the discovery by Henriques and Hansen [1903] of fatty acids in the brain more unsaturated than oleic acid, no separation of the unsaturated acids seems to have been attempted. The following preliminary separation is therefore described.

About two years ago the author observed that when bromine was added to the ether solution of the unsaturated fatty acids, the temperature being kept at 5°, a white precipitate separated which was found to contain 78–79 % bromine, and therefore corresponded to a fatty acid more unsaturated than any previously described as occurring in lipoids. The amount of such highly unsaturated acid was small, nevertheless the result will be seen to be proof of the existence of unsaturated acids of the nature of clupanodonic acid in the human brain. The bromine separation has also shown the presence of the linoleic and linolenic acid series.

Separation of the unsaturated fatty acids.

An amount of 24·1 g. of unsaturated acids was dissolved in ether and cooled to 5°, and bromine added until a definite excess was present; the solution was kept in an ice chest all night, and the precipitate (0·52 g.) separated by decantation, and washed in centrifuge tubes with ether until all bromine compounds soluble in ether were removed. An aliquot portion of the ether solution of bromides was evaporated as a check on the amount in solution. From the weight of bromides obtained it is easy to determine the percentage of bromine absorbed by the acids, which was found to be 77·5 %.

The ether was now removed from the main quantity of the soluble bromides, the latter washed free from acetic acid by hot water, and after drying shaken with ligroin of boiling point below 45°. A separation immediately followed, the larger portion being soluble while another portion was apparently insoluble in ligroin, but dissolved readily in chloroform. The quantities found together with the percentage of bromine in the fractions is set forth in the following table.

TABLE III. *The separation of 24·1 g. of unsaturated acids.*

Fraction of fatty acids	Weight of bromide found	Fatty acid which this represents	Bromine per cent.	Percentage of the acid in total unsaturated acids
Clupanodenic acid	0·52 g.	0·13	77·7	0·48
Acids of linoleic and linolenic series	5·64	2·37	58·0	9·83
Oleic acid series	34·23	21·43	37·4	88·92

Properties of the individual fractions of the unsaturated fatty acids.

(1) *Bromine compound insoluble in ether.* To the acid yielding this bromine compound I have given the name *clupanodenic acid* since it is still more unsaturated than clupanodonic acid. Although the quantity used in the analysis was small and the results must be regarded as only indicating the existence of such highly unsaturated acids, nevertheless the substance is quite definite and I have since prepared a gram of the bromide by precipitation of another quantity of unsaturated acids.

The *bromide* is a white powder, which turns a faint straw colour when heated to 120°. With further heating in a small tube it was found to blacken at about 200° and to decompose with further application of heat giving a sublimate and odour of stearic acid. Ignited on platinum foil the substance blackened and melted, burning without leaving any appreciable residue. The bromine compound is practically insoluble in ether, chloroform, and benzene, and only slightly soluble in cold alcohol. In hot alcohol it is more soluble and exhibits the properties of an acid. Titrated with alcoholic potash the molecular weight was found to be over 1000. This number was slightly low on account of the access of carbon dioxide owing to the slowness of the titration, and the titration was moreover carried out on a very small amount of material.

The bromine percentage was determined in two ways.

Data from bromine determinations.

(*a*) The substance was heated with nitric acid and silver nitrate.

0·2160 g. of bromo-acid gave 0·3946 AgBr. Br = 77·7 %.

(*b*) The substance was mixed with calcium oxide and ignited as in the method of Piria and Schiff.

0·0347 g. gave 0·0650 AgBr. Br = 79·2 %.

Calculated for dodecabromostearic acid Br = 77·9 %.

(2) *Brominated acids soluble in ether but insoluble in ligroin.* This fraction should contain any acids of the linoleic and linolenic series, and the percentage of bromine found is in agreement with this and points to the mixture consisting of both tetra- and hexa-bromine compounds. It would be premature however in view of the complexity of these lipoid fatty acids, to calculate from the bromine figure the proportions of the bromine compounds of either series of which this mixture may consist. During the process of treament with ligroin it was readily seen that at least two substances were present in the portion insoluble in that fluid for while most of the insoluble material was of a dark colour and of a plastic adhesive nature, a small portion was separated by repeated shaking and decantation which was of a light colour and amorphous. This amorphous material became of a glassy transparent nature when dried at 100° but did not melt at that temperature, whereas the main mass of the bromine compound which was insoluble in ligroin melted below 100°.

0·1556 g. of bromine compound heated with lime gave 0·2094 g. AgBr.

Br = 57·4 %.

Calculated for linoleic tetrabromide Br = 53·33 %.

„ „ linolenic hexabromide Br = 63·33 %.

(3) *Bromine compounds soluble in ether and also in ligroin.* This fraction is generally regarded as consisting of the dibromide of ordinary oleic acid. But in the present case it was found that the liquid fatty acid was admixed with a solid substance corresponding in composition to oleic acid and likewise forming a dibromide soluble in ether and ligroin. The fatty acid has not yet been examined in detail but it is evident that it occurs to a considerable extent mixed with the unsaturated liquid fatty acids of the brain and is difficult to separate from them. The acid is likewise obtained from the solid fatty acid mixture regained from the lead soaps difficultly soluble in ether, and is found left over in this separation, after fractional precipitation of the saturated acids as magnesium soaps (*vide* fraction 6, Table I).

The total bromides soluble in both ether and ligroin gave on analysis a percentage of bromine corresponding to oleic dibromide.

(*a*) 0·4507 g. gave 0·3880 AgBr. Br = 36·6 %.

(*b*) 0·4507 g. gave 0·4050 AgBr. Br = 38·2 %.

Calculated for oleic dibromide Br = 36·2 %.

Solid unsaturated fatty acid. This acid can only be briefly referred to. Further examination will be needed definitely to establish its nature. The acid was obtained from fraction No. 6 of the original magnesium soap separation, and also by exhaustion of the ether-insoluble lead soaps by hot benzene. It was purified by repeated crystallisation from acetone at a temperature of 5°. The melting point remained practically constant at 42°. The combustion figures show the substance to be of the nature of oleic acid.

(*a*) 0·1185 g. gave 0·1286 H_2O.

(*b*) 0·1323 g. gave 0·1467 H_2O; 0·3703 CO_2.

	a	*b*	Mean	Calculated for $C_{18}H_{34}O_2$	Calculated for $C_{18}H_{36}O_2$
C	—	76·34	76·34	76·60	75·66
H	12·10	12·32	12·21	12·06	12·61

The saturated fatty acids of the human brain.

The further separation of these acids obtained from fractions 2–5 of the original magnesium soap precipitation (*vide* Table I) was carried out in the usual way. The fractions were subdivided by precipitation with barium acetate and subsequently converted into silver salts which were submitted to combustion analysis. The fatty acids were regained from the silver salts and the melting points determined. In many cases the acids themselves were submitted to combustion analysis. As already pointed out, no acid of more than eighteen carbon atoms was found. The acids isolated and identified were ordinary stearic, palmitic and myristic acid, and in addition, in quantity quite equal to that of ordinary stearic acid, an isomer of stearic acid melting at 51–52°. This therefore confirms the discovery of Thudichum [1901] that the stearic acid occurring in greatest amount in the brain is not ordinary stearic acid melting at 69° but an isomer melting at 52° to which he gave the name neurostearic acid.

General Conclusions.

1. At least 25 % of the solid fatty acids of the human brain are hydroxy-acids, of which three have been isolated. Two of these at least are monohydroxy-acids and cannot therefore have been produced artificially by oxidation of unsaturated fatty acids.

2. The unsaturated acids contain besides oleic, linoleic and linolenic acids an acid still more unsaturated to which the name clupanodenic acid has been

given. It combines with twelve atoms of bromine. Such a compound has not previously been described as occurring in fats or oils.

There is present also a solid unsaturated acid melting at 42°, which is probably an isomer of oleic acid.

3. The saturated fatty acids contain normal stearic and palmitic acids and an isomer of stearic acid melting at 51–52°. The presence of myristic acid has also been confirmed.

4. In the resemblance between the hydroxy-acids of the brain, and those of lanoline, there seems to be additional evidence of the relationship of the nervous tissue to other tissues of epiblastic origin.

In conclusion I would express my thanks especially to Dr H. G. Chapman, at whose suggestion this work was begun, and to Professor T. P. Anderson Stuart, in whose laboratory the investigation was carried out.

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XV. AN INVESTIGATION OF PHYTIN.

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(*Received January 11th, 1913.*)

The chemistry of the substance phytin has attracted the attention of workers in all parts of the world. It is generally regarded as the calcium magnesium salt of inositol phosphoric acid (phytic acid), but the presence of organic constituents other than inositol has been described.

Occurrence.

Phytin occurs in the seeds of a large number of plants and was first extracted from them by Palladin [1895], who distinguished it from coagulable protein. Schulze and Winterstein [1896] prepared it by the same method and showed that it contained phosphoric acid, calcium and magnesium. They supposed it to be identical with the aleurone or globoid bodies described by Pfeffer [1872]. A better method of preparation was discovered by Posternak [1903] and he extracted the substance from various seeds. Hart and Andrews [1903] found that the phosphorus contained in the bran of cereals was chiefly in an organic form of combination, and Patten and Hart [1904] isolated a substance from wheat bran which they regarded as identical with phytin; the same substance was isolated from other cereals by Hart and Tottingham [1909] and Anderson [1912, 4] has prepared it from cotton seed meal. The distribution of phytin in various plants has been investigated by Suzuki and Yoshimura [1907], Staniszkis [1909], and Geys [1910].

Preparation.

Palladin and Schulze and Winterstein prepared phytin by extracting the fat-free seeds of *Sinapis nigra* with 10 per cent. sodium chloride solution, boiling to remove coagulable proteins and filtering when the solution had

cooled. On again boiling the solution the phytin was precipitated; it was filtered off, washed with boiling water and dried.

The method of preparation used by Posternak has been patented and phytin is now a commercial article (manufactured by the Gesellschaft für chemische Industrie in Basle). The preparation is as follows.

Oil-cake is extracted with dilute hydrochloric acid (0·5–1 per cent.) and the extract is freed from protein by one of the usual methods. Sufficient sodium acetate is added to remove free hydrochloric acid and then the necessary amount of a soluble calcium salt is added to bring the calcium content of the solution to a fixed amount. The phytin is completely precipitated with copper acetate as a complex copper, calcium and magnesium salt. The precipitate is washed with distilled water and decomposed with hydrogen sulphide. The filtrate from the copper sulphide is evaporated *in vacuo* to a syrup, treated with alcohol and the solid mass dried and powdered.

The extract may be freed from impurities by oxidation, and organic acids such as formic may be used instead of hydrochloric acid.

Phytic acid may be prepared by extracting acidified solutions of phytin with a mixture of one volume of alcohol and three volumes of ether. A syrup is obtained on evaporation which may be precipitated as copper salt as before.

Acid salts of phytic acid may be prepared by dissolving phytin in dilute mineral acids and precipitating with alcohol; with just sufficient acid to dissolve the phytin the precipitate is insoluble in water, but if more acid be used a precipitate of the soluble acid salt of the type CaH_2X is obtained; with still more acid the precipitate is the acid salt of the type CaH_6X.

Composition of Phytin.

The method of preparation of phytin from extracts of seeds and bran does not exclude the presence of calcium and magnesium phosphate in the preparations which have been made.

There are many conflicting statements concerning the precipitation of phytin by ammonium molybdate; some authors have stated that it is precipitated, some that it is not precipitated; some have said that it gives a white precipitate, others a yellow precipitate. The yellow precipitate has been attributed by many observers to impurities in the phytin. Starkenstein [1910] claimed to have shown that phytin contained inorganic phosphate and that it was decomposed by drying at 100°, but Jegoroff [1912] showed the contrary. Jegoroff pointed out that there is no satisfactory method for

estimating inorganic phosphate in the presence of phytic acid though in some cases that of Schulze and Castoro had been of use. Fingerling and Hecking [1911] have also complained of the absence of a suitable method for the estimation of inorganic phosphate in extracts of fodders containing phytic acid.

None of the investigators mention the methods by which they have ascertained the calcium, the magnesium and the phosphoric acid content of phytin. Most of the methods for the estimation of calcium and magnesium in the presence of phosphoric acid are long and involve possibilities of error. This may account for the very different values which have been found, e.g.:

	Ca	Mg	P	Na	K	Observer
CaMg salt ...	—	—	15·13	—	—	Schulze and Winterstein.
Mg salt	—	7·78	18·44	—	—	Winterstein.
CaMg salt ...	13·42	8·05	22·30	—	—	Posternak [1900]*.
CaNa salt ...	8·41	—	19·42	18·79	—	,, [1903].
,, ...	8·16	—	19·13	19·02	—	,, ,,
CaMgK salt (wheat)	1·13	5·80	16·38	—	2·60	Patten and Hart.
,, (maize)	0·48	6·70	14·17	—	5·60	Hart and Tottingham.
,, (oats)	8·60	4·70	16·70	—	1·50	,, ,,
,, (barley)	0·00	7·90	14·46	—	11·20	,, ,,
,, (rice)	5·18	17·48	23·48	—	trace	Suzuki and Yoshimura.
CaMg salt (commercial)	11·96	1·45	20·32	—	—	Horner.
CaMgK salt (from phytic acid)	13·03	4·29	19·09	—	6·42	Anderson [1912, 2].
CaMgK salt (wheat bran)	2·82	4·72	14·42	—	—	,, [1912, 3].

The carbon, hydrogen and phosphorus content of phytin and of phytic acid as given by various workers are also very different:

	C	H	P	Observer
Phytic acid	9·87	3·70	25·89	Posternak.
,, ...	9·97	3·65	26·00	,,
,, (wheat) ...	10·63	3·38	25·98	Patten and Hart.
,, (maize) ...	10·30	—	26·07	Hart and Tottingham.
,, (oats) ...	10·22	—	25·97	,, ,,
,, (barley) ...	10·54	—	25·88	,, ,,
,, (cotton seed)	11·71	3·07	26·35	Anderson [1912, 4].
,, ,,	10·89	3·25	27·11	,, ,,
,, ,,	11·02	3·30	26·36	,, ,,
Calculated for $C_6H_{24}O_{27}P_6$	10·08	3·36	26·05	——
CaMg salt	9·65	2·88	15·13	Schulze and Winterstein.
CaNa salt	7·25	1·34	19·42	Posternak.
,, ...	7·43	1·49	19·13	,,
CaMgK salt (wheat bran)	17·30	3·63	16·38	Patten and Hart.
,, ,,	21·47	3·69	14·42	Anderson [1912, 3].

* Cited in Jegoroff's paper.

The difficulty of burning all the carbon in the presence of phosphoric acid has never been mentioned and the different values may be assigned to this cause.

The Preparation of Phytic Acid.

Phytic acid was prepared by Posternak, who described it as a thick transparent brownish syrup. Patten and Hart again prepared phytic acid from the phytin of the bran of wheat. They pointed out the great difficulty in obtaining it free from calcium (compare the precipitation of phytin with copper acetate) and they adopted the following procedure. The copper compound which is precipitated from the extract is decomposed by hydrogen sulphide. The filtered solution is made alkaline with sodium hydroxide and precipitated with barium chloride. The barium salt is washed until free from alkali, suspended in water and decomposed with the exact amount of sulphuric acid to combine with the barium. The solution is again precipitated with barium chloride in alkaline solution and treated as before. The process is once more repeated and then copper acetate is added. The copper compound which is precipitated is washed, suspended in water and decomposed with hydrogen sulphide. The filtrate from the copper sulphide is evaporated to a syrup and the residue is dried at 110°.

Constitution of Phytic Acid.

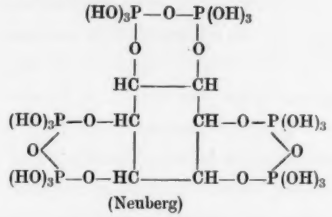
The evidence for the constitution at present accepted for phytic acid is based upon the work of Winterstein and of Posternak. Winterstein [1897] prepared a magnesium salt of phytic acid and obtained on hydrolysis with concentrated hydrochloric acid at 130° a 60 per cent. yield of inositol. Posternak analysed the acid and determined its molecular weight by the freezing point method. He gave it the formula $C_2H_8O_9P_2$ and represented it as anhydro-oxymethylene diphosphoric acid,

$$O\begin{cases}CH_2—O—PO(OH)_2\\CH_2—O—PO(OH)_2.\end{cases}$$

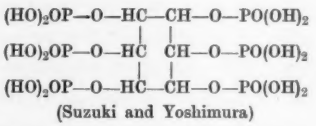
He confirmed the observation of Winterstein that it yielded inositol on hydrolysis (98 per cent. yield) and explained the formation of this product by the polymerisation of the formaldehyde which is first formed.

This constitution of phytic acid was challenged by Neuberg [1908, 1] who quoted Suzuki and Yoshimura's result that inositol is obtained by hydrolysis with the enzyme, phytase. Winterstein [1908] was also of opinion that the inositol nucleus existed in phytic acid. Neuberg and Suzuki and Yoshimura

[1907] put forward formulae representing phytic acid as inositol hexaphosphoric acid:



(Neuberg)



(Suzuki and Yoshimura)

Contardi [1909] contributed further evidence to show that phytic acid is a phosphoric acid ester of inositol: he obtained 18 g. of inositol from 100 g. of phytin by acid hydrolysis.

Starkenstein [1910] from titrations of phytic acid supposed that phytic acid was derived from pyrophosphoric acid and Vorbrodt [1910], who analysed a "sphero" crystalline barium salt, believed that the phytic acid molecule contained two inositol nuclei.

The synthesis of esters of phosphoric acid and inositol has been effected by Contardi [1910] and by Anderson [1912, 1]. Contardi heated together inositol and phosphoric acid and obtained inositol hexaphosphoric acid together with a quantity of inositol diphosphoric acid. The inositol hexaphosphoric acid agreed very closely in its properties with natural phytic acid. By the same method Anderson obtained inositol tetraphosphoric acid as the chief product of the reaction; inositol hexaphosphoric acid was not formed. Anderson [1912, 2] has also prepared an ester of inositol and pyrophosphoric acid. Carré [1911] has maintained that the substances prepared by Contardi are mixtures of inositol and phosphoric acid, but Contardi [1912] refuted these statements.

Most observers seem to agree that phytic acid is composed of only inositol and phosphoric acid, but the data concerning the yield of inositol obtained on hydrolysis do not support this conclusion. Posternak alone obtained a nearly theoretical yield; other workers have obtained yields varying from 20 to 75 per cent. and in the majority of cases the yield has not been given. Levene [1909] claimed to have separated phytin into two constituents; one of them yielded inositol on hydrolysis; the other a carbohydrate which gave pentose reactions and furfural on distillation with hydrochloric acid. Neuberg [1909] severely criticised the claims of Levene and showed that inositol gave small quantities of furfural when distilled with concentrated hydrochloric acid. Considering the scanty data of the yield of

inositol on hydrolysis, Levene's claim that another organic constituent is present in phytin must be considered. Anderson [1912, 3] finds that the phytin of bran does not consist of one substance, but is a mixture.

The above summary of the work on phytin shows that there are several lines of investigation which require attention:

(1) The presence of inorganic phosphates in phytin.

(2) The analysis of the calcium and magnesium content of phytin.

(3) The removal of calcium in the preparation of phytic acid.

(4) The yield of inositol by the hydrolysis of phytin or phytic acid.

(5) The presence of other organic compounds besides inositol in phytic acid.

Our investigations are not complete but we believe we have sufficient data to justify their publication, especially as our joint work has ceased for some time, although we hope to continue it at some future date.

Experimental.

Commercial phytin has been employed almost entirely in these investigations, but for comparison phytin (neutral calcium phytate) was prepared from bran. 200 g. of wheat-bran were extracted with successive quantities of N/5 hydrochloric acid until no appreciable amount of phosphorus was contained in the extract. The combined extracts were made alkaline with ammonia; this caused the precipitation as neutral calcium phytate of the whole of the calcium in the extract. The residual phytic acid in the solution was precipitated by adding calcium chloride solution until no further precipitation ensued. The whole was then filtered and the precipitate thoroughly washed with water and dried. The product was dissolved in dilute hydrochloric acid and again thrown down by adding ammonia. The precipitate was re-dissolved in hydrochloric acid and the solution decolourised by shaking with charcoal. After filtering, the phytin was precipitated with ammonia and filtered off. It was washed four times by removing from the filter, triturating with distilled water and filtering, and then dried at 100°. Yield = 6 g. or 3 per cent.

Analysis of Phytin.

(i) *Estimation of the Total Phosphorus.* The total phosphorus content of phytin was estimated by the method of Neumann as modified by Plimmer and Bayliss [1906]. Several samples were analysed. The analytical data are all given on p. 168.

(ii) *Estimation of Inorganic Phosphorus.* Inorganic phosphate in the presence of organic phosphates can be readily estimated by precipitation with ammonium magnesium citrate as ammonium magnesium phosphate and conversion into magnesium pyrophosphate as was shown by Plimmer and Bayliss in the case of caseinogen. The same method can be applied to the estimation of inorganic phosphate in the presence of glycerophosphate and ethyl phosphate, as was shown by one of us in a previous communication, but in the presence of phytic acid this method cannot be employed. The solution of phytic acid obtained after removal of the calcium by oxalic acid or an oxalate not only inhibits the precipitation of small amounts of inorganic phosphate as ammonium magnesium phosphate but also, when larger amounts are present, renders the precipitation slow and the precipitate consists of ammonium magnesium phosphate and ammonium magnesium phytate. This is shown by the following experiments.

Various amounts of a solution of sodium phosphate were added to a solution of phytic acid prepared by adding oxalic acid to 10 g. of phytin dissolved in water, filtering and diluting to one litre.

A. 10 c.c. phytic acid sol. + 1 c.c. Na_2HPO_4 sol. gave no ppt.
,, ,, + another 1 c.c. Na_2HPO_4 sol. gave no ppt.
,, ,, + yet another 1 c.c. Na_2HPO_4 gave ppt. = 0·0692 g. P_2O_5.

						Hence ppt. from phytic acid
B.	10 c.c. phytic acid sol.	+ 1 c.c.	Na_2HPO_4 sol. gave ppt.	= 0·0313 g.	P_2O_5.	= 0·0072
	,, ,,	+ 2 c.c.	,, ,,	= 0·0629	,,	= 0·0147
	,, ,,	+ 3 c.c.	,, ,,	= 0·0925	,,	= 0·0202
		1 c.c.	,, ,,	= 0·0241	,,	
C.	10 c.c. phytic acid sol.	+ 5 c.c.	Na_2HPO_4 sol. gave ppt.	= 0·1187 g.	P_2O_5.	= 0·0075
	,, ,,	+ 5 c.c.	,, ,,	= 0·1350	,,	= 0·0238
		5 c.c.	,, ,,	= 0·1112	,,	

The estimation of inorganic phosphate in the presence of phytic acid was carried out by Hart and Andrews [1903] by precipitation with ammonium molybdate at 65° in the presence of very dilute nitric acid. This method was used by McCollum and Hart in their phytase experiments. Under these conditions there is a possibility that phytic acid is hydrolysed with the liberation of inorganic phosphate. Experiments were therefore made to ascertain if inorganic phosphate was completely precipitated by ammonium molybdate in the presence of seminormal to normal nitric acid at room temperature. The yellow solution which is first formed on adding ammonium molybdate to a solution of sodium phosphate in the presence of dilute nitric acid gradually deposits ammonium phosphomolybdate in a crystalline state and the solution becomes colourless in 24 to 48 hours. On determining the

amount of P_2O_5 in this precipitate by the Neumann method, by dissolving it, after filtration and washing, in semi-normal sodium hydrate, boiling off the ammonia and titrating with semi-normal sulphuric acid it was found to be quantitative:

1 c.c. Na_2HPO_4 sol. gave $Mg_2P_2O_7 = 0{\cdot}0241$ g. P_2O_5.
1 c.c. ,, ,, + 25 c.c. H_2O + 25 c.c. 2N . HNO_3 + 25 c.c. Am. molybdate sol. } gave { 0·0226 g. P_2O_5. 0·0219 ,,

Applying this method to the solution of phytic acid the following amounts were obtained:

10 c.c. phytic acid sol. + 25 c.c. 2N . HNO_3 + 25 c.c. H_2O + 10 c.c. Am. molybdate sol. } gave { 0·0043 g. P_2O_5. 0·0042 ,, 0·0044 ,,

Total P_2O_5 in 10 c.c. phytic acid sol. by Neumann's method = 0·0871 gm.

Further proof that the estimation of inorganic phosphate in the presence of phytic acid can thus be effected is given by the following experiments which were carried out in the same way:

(*a*) 1 c.c. Na_2HPO_4	(*b*) 10 c.c. phytic acid sol.	(*c*) 1 c.c. Na_2HPO_4 + 10 c.c. phytic acid sol.	Sum of (*a*) and (*b*)
0·0223 g. P_2O_5	0·0044 g. P_2O_5	0·0264 g. P_2O_5	0·0267
0·0226 ,,	0·0054 ,,	0·0270 ,,	0·0281
0·0222 ,,	0·0051 ,,	0·0270 ,,	0·0273

Hydrolysis of the phytic acid by dilute nitric acid at room temperature therefore does not occur. Employing this method of estimation it has been shown that hydrolysis does not occur at 37° but at 65° and at 75° phytic acid is slowly hydrolysed [Plimmer, 1913, 2, p. 79].

If proteins be present in solution they are precipitated by phosphomolybdic acid with the ammonium phosphomolybdate. The estimation can be carried out by filtering the yellow precipitate, washing free from acid, dissolving in sodium hydrate and then precipitating in the usual way with magnesium citrate mixture and ammonia and finally converting into magnesium pyrophosphate.

The above three solutions after the estimation by Neumann's method were filtered and the inorganic phosphate estimated as magnesium pyrophosphate:

(*a*)	(*b*)	(*c*)	(*a*) + (*b*)
0·0239 g. P_2O_5	0·0042 g. P_2O_5	0·0281 g. P_2O_5	0·0281
0·0240 ,,	—	0·0290 ,,	—
0·0239 ,,	0·0050 ,,	0·0289 ,,	0·0289

The estimation of inorganic phosphate in phytin can be readily effected by this method; the presence of calcium does not interfere with the

precipitation. A known weight of phytin is dissolved in normal nitric acid, excess of ammonium molybdate is added and the solution is allowed to stand. When the solution is colourless the crystalline precipitate is filtered off and estimated by the Neumann method, or by filtering, washing free from acid, dissolving in sodium hydrate and precipitating as ammonium magnesium phosphate.

The results of the estimations in different samples of phytin are given on p. 168.

(iii) *Estimation of Calcium and Magnesium.* The previous investigators have not stated the method which they employed for the estimation of these elements in phytin. They have in all probability precipitated the calcium as oxalate and converted it into calcium oxide, either before or after oxidation of the organic matter, and estimated the magnesium in the filtrate as magnesium pyrophosphate. This method involves several possibilities of error; if phosphoric acid be not removed it may be thrown down with the calcium oxalate and some of the magnesium may also be precipitated as magnesium oxalate. The method of separation of these constituents in a mixture is again a long and tedious process.

If oxalic acid or an oxalate be added to a solution of phytin a precipitate is formed, but this precipitate does not consist of pure calcium oxalate. This is shown by the following experiment:

10 g. of commercial phytin were dissolved in decinormal acetic acid and filtered from a small quantity of insoluble matter. This was dried at 110° and analysed: it contained 13·74 per cent. of calcium (estimated as $CaSO_4$, see later) and 17·4 per cent. of phosphorus (by Neumann's method). The filtrate was precipitated with 4·1 g. of oxalic acid dissolved in 50 c.c. of water. The precipitate was washed and dried at 110°; it weighed 5 g. and was analysed:

0·2188 g. heated to redness to constant weight gave 0·0956 g. residue. Calculated amount for pure calcium oxalate is 0·0957 g.
0·3364 g. gave 0·2782 g. $CaSO_4$ (see later) and 0·0031 g. P by Neumann's method.
0·3030 g. gave 0·0028 g. P.

Ca = 24·31 per cent. $(COO)_2Ca$ requires Ca = 31·25 per cent.
P = 0·92 per cent.
P = 0·93 per cent.

Another preparation of calcium oxalate from phytin contained 0·77 per cent. total P and 0·27 per cent. inorganic P.

Calcium can therefore not be estimated by precipitating with oxalic acid.

Aron [1907] showed that the calcium in milk, urine, etc., could be estimated as calcium sulphate after oxidation of the organic matter by the

Neumann method. When the oxidation is completed the nitric acid is removed as far as possible by diluting with water and again evaporating. The sulphuric acid which remains is then diluted and four volumes of alcohol are added. The calcium sulphate is precipitated and is filtered off, dried and weighed.

Beyond these few estimations of calcium Aron made no other analyses, but he states that other elements might be estimated in the filtrate.

This method therefore required verification and extension for the estimation of magnesium. Analyses of mixtures of calcium chloride, magnesium sulphate and sodium phosphate were consequently made to test the method. The mixtures were evaporated down in a small conical flask with 10 c.c. of sulphuric acid; the acid was diluted with water and precipitated with four to five volumes of alcohol. The calcium sulphate was filtered off on a Gooch crucible, and dried at a red heat. The magnesium was estimated in the filtrate as pyrophosphate after evaporating off the alcohol, oxidising the remainder of the alcohol with nitric acid, diluting with water and making alkaline with ammonia. Excess of phosphoric acid was present in the mixtures to precipitate the whole of the magnesium. The results were:

Calcium		Magnesium	
Taken	Found	Taken	Found
0·1736	0·2540	0·1484	0·1490
0·1736	0·2066	0·0297	0·0310
0·0347	0·0523	0·1484	0·1493
0·1736	0·1885	0·1484	0·1495
0·0264	0·0569	0·0337	0·0332
0·0264	0·0328	0·0337	0·0332
0·0264	0·0588	0·0337	0·0329

Magnesium can thus be correctly estimated but the values for calcium are too high. The high values are due to the presence of sodium in the solution as is shown by experiments with mixtures of calcium chloride and sodium phosphate and calcium chloride and phosphoric acid in various proportions:

Calcium			Calcium		
Taken	Found	In presence of sodium phosphate	Taken	Found	In presence of phosphoric acid
0·0233	0·0285	5 c.c.	0·0233	0·0233	10 c.c.
	0·1038	10 c.c.		0·0233	15 c.c.
	0·1944	15 c.c.		0·0232	20 c.c.
	0·2740	20 c.c.		0·0231	25 c.c.

The presence of sodium salts in the solution thus causes a serious error in the estimation of calcium as calcium sulphate.

If the amount of alcohol used in the precipitation be reduced and if the amount of sodium or potassium in the solution be not greater than 0·1 g. the

estimation of calcium can be accurately effected, especially if not more than one volume of alcohol be used in the precipitation:

3 volumes alcohol		2 volumes alcohol		1 volume alcohol	
Calcium found	Amount of Na present, in g.	Calcium found	Amount of K present, in g.	Calcium found	Amount of K present, in g.
0·0231	0·0	0·0232	0·0	0·0231	0·0
0·0231	0·0	0·0245	0·0394	0·0228	0·0
0·0284	0·1228	0·0281	0·0591	0·0230	0·0197
0·0268	0·0614	0·0383	0·0788	0·0230	0·0394
0·0258	0·0307	0·0237	0·0197	0·0228	0·0591
0·0238	0·0184	0·0238	0·0158	0·0230	0·0788
0·0233	0·0123	0·0237	0·0118	0·0227	0·0788

The amount of calcium taken in all these experiments was 0·0233 g.

Unless the amount of potassium and sodium (if present) in phytin exceed 20 per cent. (0·1 g. in 0·5 g. required for an analysis) the calcium can be accurately estimated by precipitation with one volume of alcohol as calcium sulphate and the magnesium can be estimated in the filtrate as magnesium pyrophosphate. The calcium and magnesium content of various samples of phytin have been estimated by oxidising the organic matter with 10 c c. sulphuric acid and nitric acid, removing the nitric acid by diluting and evaporating, adding 10–20 c.c. of water and then one volume of alcohol (20–60 c.c.) to precipitate the calcium sulphate. Alcohol was removed from the filtrate by evaporation. On oxidation with nitric acid and on making alkaline with ammonia the magnesium was precipitated as ammonium magnesium phosphate and estimated as pyrophosphate. Excess of P_2O_5 is always present in phytin: the remainder can be estimated by adding magnesia mixture and determining it as pyrophosphate. The sum of the figures obtained then gives the total P_2O_5 content. It has been found to be the same as by the Neumann method. For the data see p. 168.

(iv) *Loss of Weight on Heating and* in vacuo *over Sulphuric Acid.* Phytin loses weight at 110° and *in vacuo* over sulphuric acid. The greater part of the loss takes place during the first heating; the weight continues to decrease very slowly and loss of weight seems to go on indefinitely. One specimen was heated at 110° for 54 hours. The loss in weight under both conditions is about the same, but it takes place more slowly *in vacuo.*

Part of the loss in weight may be due to alcohol; one of the commercial specimens examined gave the iodoform reaction.

The gradual decrease in weight on heating is probably due to a slow conversion into pyrophosphate.

(v) *Estimation of Carbon and Hydrogen.* In the course of many combustions of organic phosphorus compounds the difficulty of burning all the

carbon has been noticed. Page [1912] has shown that the values for carbon in the case of the hydroxyphosphinic acids were 1–2 per cent. below the true value even after mixing the substance intimately with lead chromate. The values were still lower by the Fritsch-Messinger wet method of combustion.

Previous observers have never mentioned any difficulty in ascertaining the carbon content of phytin or other organic phosphorus compounds nor have they mentioned any modification of the ordinary method which they used.

One of the specimens of phytin was analysed by mixing intimately with the finest copper oxide and it seems improbable that all the carbon was not burnt.

Results of Analyses.

Total Phosphorus.

Commercial phytin:			
Sample 1	0·1120 g.	37·8 c.c. $\frac{N}{2}$ NaOH.	18·68 per cent.
2	0·0682 g.	24·7 c.c. ,,	20·04 ,,
3			17·74 ,,
4	0·0579 g.	18·7 c.c. ,,	18·57 ,,
5	0·0694 g.	24·1 c.c. ,,	19·22 ,,
6	0·0614 g.	21·2 c.c. ,,	19·11 ,,
7	0·0544 g.	18·7 c.c. ,,	19·76 ,,
8	0·0550 g.	18·5 c.c. ,,	18·62 ,,
Bran (1)	0·6704 g.	15·8 c.c. ,,	1·30 ,,
Phytin from bran	0·1326 g.	0·0714 g. $Mg_2P_2O_7$*	15·00 ,,

* Estimated by Mg method after ppt. of $CaSO_4$.

Inorganic Phosphorus.

Commercial phytin:			
Sample 2	0·2664 g.	4·3 c.c. $\frac{N}{2}$ NaOH.	0·89 per cent.
4	0·6152 g.	10·8 c.c. ,,	0·97 ,,
5	0·9642 g.	17·1 c.c. ,,	0·98 ,,
6	0·6626 g.	11·7 c.c. ,,	0·98 ,,
7	0·5350 g.	9·5 c.c. ,,	0·98 ,,
8	0·8726 g.	14·8 c.c. ,,	0·94 ,,
Phytin from bran	0·3524 g.	16·7 c.c. ,,	2·62 ,,

Calcium and Magnesium.

				Per cent.	
Commercial phytin:				Ca	Mg
Sample 1	0·4552 g.	0·1984 g. $CaSO_4$	0·0092 g. $Mg_2P_2O_7$.	12·82	0·44
2	0·5630 g.	0·2340 g. ,,	0·0194 g. ,,	12·2	0·75
3	0·4090 g.	0·1828 g. ,,	0·0104 g. ,,	13·15	0·56
4	0·5235 g.	0·2207 g. ,,	0·0195 g. ,,	12·40	0·81
5	0·4245 g.	0·1744 g. ,,	0·0155 g. ,,	12·10	0·78
6	0·4224 g.	0·1775 g. ,,	0·0151 g. ,,	12·36	0·78
7	0·4052 g.	0·1681 g. ,,	0·0151 g. ,,	12·20	0·81
8	0·4466 g.	0·1873 g. ,,	0·0155 g. ,,	12·34	0·76
Phytin from bran	0·1326 g.	0·0962 g. ,,	0·0000 g. ,,	21·12	0·0
Bran (1)	3·3430 g.	0·0150 g. ,,	0·0786 g. ,,	0·13	0·51
(2)	4·4868 g.	0·0176 g. ,,	0·1116 g. ,,	0·11	0·54
(3)	4·2028 g.	0·0228 g. ,,	0·1058 g. ,,	0·15	0·55

Loss of Weight (a) at 110° and (b) in vacuo over H_2SO_4.

Commercial phytin:		
Sample 3 (*a*)	5·0229 g. : 0·3425 g. = 6·82	per cent.
3 (*b*)	5·6868 g. : 0·3874 g. = 6·81	,,
4 (*a*)	0·7376 g. : 0·0544 g. = 7·37	,,
5 (*a*)	1·7712 g. : 0·1510 g. = 8·52	,,
6 (*a*)	1·5502 g. : 0·1164 g. = 7·51	,,
7 (*a*)	0·8472 g. : 0·0628 g. = 7·41	,,
8 (*a*)	2·1870 g. : 0·1532 g. = 7·00	,,

Carbon and Hydrogen.

Commercial phytin:		
Sample 2	0·3052 g. : 0·0815 g. CO_2 : 0·0917 g. H_2O.	C = 7·28 %, H = 3·33 %.
	0·2392 g. : 0·0655 g. ,, : 0·0740 g. ,,	C = 7·47 %, H = 3·44 %.

If these figures be compared with those of previous workers (p. 159) considerable differences will be noticed. These figures agree most closely with those of Horner for commercial phytin and of Anderson for the preparation made from phytic acid. The total phosphorus content is generally higher and so is the calcium content, but the magnesium content is much lower; in all the specimens examined magnesium was less than one per cent. Commercial phytin seems to be the calcium salt and the magnesium may be present as impurity.

The carbon content is also lower, but the hydrogen content agrees with the analyses of other workers.

The commercial preparations are very constant in their phosphorus, calcium and magnesium content, but they differ from the preparation from wheat bran, which was the neutral calcium salt of phytic acid and hence contained less phosphorus and more calcium; the bran preparation contained no magnesium.

Analyses of the phosphorus, calcium and magnesium in wheat bran were made for comparison. It will be observed that bran contains more magnesium than calcium, which is the reverse of phytin.

Preparation of Phytic Acid.

Phytic acid was prepared from commercial phytin and from the phytin from bran by the method of Patten and Hart and also by the removal of the calcium as oxalate. Neither method sufficed to remove the calcium entirely, and it was at first thought that some hitherto undescribed product was present.

Preparation 1. 50 g. of commercial phytin (sample 3 above) were

dissolved in a minimum quantity of dilute hydrochloric acid and just sufficient sodium acetate solution was added to render the solution neutral to Congo-red paper. Copper acetate solution was then added until no further precipitation occurred, and the copper salt filtered off by suction. Since it was impossible to wash the precipitate thoroughly on the paper it was removed, made into a uniform cream with distilled water and passed through muslin to ensure the complete breaking up of all lumps. The mass was filtered and the washing procedure repeated twice. (All subsequent washings were effected in this way.) The washed copper phytate precipitate was suspended in water and the mixture treated with hydrogen sulphide until saturated and set aside for twelve hours. The copper sulphide was filtered off and washed. The filtrate and washings were exactly neutralised with sodium hydroxide and excess of barium chloride solution added. The precipitate of barium salt was filtered off and washed twice as described above. It was then suspended in water and the exact quantity of sulphuric acid necessary to combine with the whole of the barium was added, and the barium sulphate filtered off and washed. The solution was neutralised with sodium hydrate and the whole procedure of precipitation with barium chloride and decomposition with sulphuric acid repeated twice. The solution after removal of the barium for the third time was neutralised with caustic soda and then acidified with acetic acid. Copper acetate solution was added and the copper salt so obtained filtered off, washed and decomposed as described above. The filtrate from the copper sulphide was evaporated to a syrup at 100°.

Preparation 2. A specimen of phytic acid was prepared in the same manner from 5 g. of the phytin from bran.

Preparation 3. 100 g. of commercial phytin (sample 4 above) were dissolved as far as possible in decinormal acetic acid. The insoluble residue (amounting to 4 g.) was filtered off and washed. To the filtrate was added the calculated quantity of oxalic acid (37·5 g. allowing for the residue, which contained 11·81 per cent. of calcium) dissolved in water. The calcium oxalate was filtered off and a portion of the filtrate tested with a little more oxalic acid to ascertain if the calcium had been completely removed; no further precipitate was produced. Since calcium oxalate is known to occlude magnesium oxalate to a considerable degree it was thought that the precipitated calcium oxalate would remove at the same time the small quantities of magnesium present. The filtrate was therefore evaporated to a small volume *in vacuo*; a small amount of calcium oxalate separated out and was filtered off. The solution was then concentrated to a syrup at 100°.

The presence of calcium in the syrups of phytic acid.

In the case of all three preparations of phytic acid it was noticed that after drying at 100° an appreciable scum or skin formed on the surface and that when small samples of each specimen were treated with alcohol a small white flocculent precipitate was formed. Each of the preparations was therefore dissolved in a small quantity of water and sufficient alcohol added to precipitate completely the insoluble substance. This was filtered off, washed thoroughly with alcohol, re-dissolved in water and re-precipitated with alcohol. It separated as a white flocculent precipitate which rapidly became stringy and formed a sticky mass on the bottom of the vessel; on standing under alcohol for some time it became quite hard and apparently crystalline. The yields were: (1) 3 g., (2) 1·3 g., (3) 12 g.

On analysis when dried at 110° these precipitates were found to contain phosphorus and calcium, and the third preparation magnesium as well:

Prep. 1	0·2162 g.	: 0·0883 g. $CaSO_4$	: Ca = 12·01 per cent.
2	0·2197 g.	: 0·1004 g. ,,	: Ca = 13·45 ,,
3	0·2586 g.	: 0·0654 g. ,,	: Ca = 7·46 ,,
		0·0357 g. $Mg_2P_2O_7$	: Mg = 3·12 ,,
Prep. 1	0·0527 g.	: 21·10 c.c. $\frac{N}{2}$ NaOH	: P = 20·79 per cent.
2	0·0227 g.	: 8·95 c.c. ,,	: P = 21·80 ,,
3	0·0304 g.	: 11·80 c.c. ,,	: P = 21·50 ,,

Another preparation by the oxalic acid method was found to contain 21·1 per cent. of phosphorus, 11·10 per cent. of carbon and 2·98 per cent. of hydrogen.

The alcoholic filtrates were evaporated to syrups at 100°. They agreed in most of their properties with the descriptions of other workers.

When dried to constant weight at 100° and *in vacuo* over sulphuric acid for three months the syrup from Preparation 1 was analysed:

Prep. 1 0·3444 g. : 0·1498 g. CO_2 : 0·1108 g. H_2O. C = 11·86, H = 3·57 per cent.
0·0986 g. : 38·0 c.c. $\frac{N}{2}$ NaOH. P = 21·34 per cent.

$(C_2H_8O_9P_2)_3$ requires C = 10·09 and H = 3·36 per cent. P = 26·07 per cent.

The carbon and hydrogen figures are higher than those required for phytic acid whilst the phosphorus figure is much lower. It is very probable that some alcohol is retained in the syrup and is not removed by drying; it is also possible that some esterification took place between the alcohol and the phytic acid.

Formation of Inositol by Hydrolysis of Phytic Acid.

The hydrolysis of phytin by dilute nitric acid at the atmospheric pressure and at various temperatures has been investigated by one of us [Plimmer, 1913, 2]; even after seventeen days the separation of the phosphoric acid was not quite complete.

64 g. of phytic acid (the syrup after removal of the crystalline calcium compound in Preparation 3, prepared from 100 g. phytin by treatment with oxalic acid) were dissolved in 250 c.c. of twice normal sulphuric acid and heated in a corked flask in a boiling water bath for 36 hours; about 55 per cent. of the phosphorus was then present as inorganic phosphate; the heating was continued at 75° in a thermostat for 164 hours; the solution now contained 79 per cent. of the phosphorus in an inorganic form. The hydrolysis was proceeded with for another 118 hours after diluting with four volumes of twice normal sulphuric acid. 84 per cent. of the total phosphorus was now present as phosphoric acid. Since the hydrolysis proceeded so slowly it was discontinued. The solution was evaporated to 200 c.c. and an equal volume of alcohol was added, followed by ether until a permanent cloudiness was produced. Inositol crystallised out when the mixture was set aside at 0° for twenty-four hours and was removed by filtration. Two further quantities were obtained on adding ether to the filtrate and keeping at 0°. The total yield amounted to 4·5 g. The alcohol and ether were distilled from the solution and the volume made up to 500 c.c. This solution contained 10·38 g. total phosphorus and 9·11 inorganic phosphorus, i.e. 1·27 g. organic phosphorus or 5·08 g. of phytic acid (25 per cent P). Allowing for the samples removed during hydrolysis the liquid from which the inositol was isolated contained 48 g. of phytic acid, and allowing for the organic phosphorus still present the amount of phytic acid hydrolysed is 42 g. This quantity should yield 10·06 g. of inositol. Since the amount actually obtained was only 4·5 g. it represents less than 50 per cent. of the theoretical.

The inositol was recrystallised from water, alcohol and ether; it melted at 220–222°. Analysis:

0·2140 g.	: 0·2550 g. CO_2	: 0·1230 g. H_2O	: C = 32·5 per cent., H = 6·4 per cent.
$C_6H_{12}O_6$ requires	...	...	C = 40·0 per cent., H = 6·7 per cent.

The figure for carbon is too low. A residue was left in the boat and amounted to 7 per cent.; it contained particles of carbon which it was impossible to oxidise completely after many hours in a current of oxygen.

This combustion again shows the difficulty of burning carbon in the presence of phosphoric acid. The ash was found to contain 52·14 per cent. of P_2O_5.

Summary.

1. Inorganic phosphates in phytin can be readily estimated by precipitation with ammonium molybdate in semi-normal nitric acid at room temperature.

2. The calcium content of phytin cannot be estimated by precipitation as calcium oxalate, but is easily ascertained by precipitation as calcium sulphate.

The magnesium content of phytin can then be estimated as magnesium pyrophosphate.

3. There is great difficulty in removing the calcium from phytin in the preparation of phytic acid.

4. The yield of inositol obtained on the hydrolysis of phytic acid by acids is not quantitative; we are inclined to believe that another organic constituent is present in phytin, and hope to carry out further experiments on the production of inositol by the hydrolysis of phytic acid at some future date.

The expenses of this work were partly defrayed by a grant from the Government Grant Committee of the Royal Society.

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XVI. ON THE RELATIONS OF THE PHENOLS AND THEIR DERIVATIVES TO PROTEINS. A CONTRIBUTION TO OUR KNOWLEDGE OF THE MECHANISM OF DISINFECTION.

PART II. A COMPARATIVE STUDY OF THE EFFECTS OF VARIOUS FACTORS UPON THE GERMICIDAL AND PROTEIN-PRECIPITATING POWERS OF THE PHENOLS.

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(*Received January 13th, 1913.*)

Evidence set forth in a previous communication on this subject [Cooper, 1912, 2] suggested that the germicidal power of the phenols is due, not to a typical chemical action upon the bacterial proteins, as appears to be the case with formaldehyde, but to a de-emulsifying effect upon their colloidal suspensions as evidenced by the precipitation of proteins when a certain phenol concentration is attained.

Since H. Chick [1910] has shown that disinfection by heat is analogous to the heat-coagulation of proteins, it seems that a similarity in mechanism exists between the germicidal actions of heat and of the phenols.

Hardy [1899] pointed out that the heat-coagulation of proteins consists of two processes, namely, (1) The reaction between the protein and hot water (denaturation). (2) The subsequent separation of the altered protein in a particulate form (agglutination). It is not at present known whether the essential stage in the processes of disinfection by heat and the phenols is a denaturation of the bacterial proteins or a destruction of their colloidal suspension.

The experimental work described in this paper has been undertaken with the object of studying more closely the apparent relationship between the bactericidal action of the phenols and their precipitating effects on proteins.

I. The Influence of Chemical Constitution upon the Germicidal and Protein-Precipitating Action of the Phenols.

The experimental method. The minimum concentrations in water of various phenols and certain other substances have been determined at which a visible change in the state of aggregation of the protein occurred. Small alterations in the magnitude of the protein-particles brought about by such concentrations were indicated by a turbidity; larger alterations by a progressively increasing precipitation of the colloid as the concentrations of the phenols rose.

Two proteins were used in the experiments, namely, gelatin and dialysed egg-albumin; the former was employed in the form of small strips and the latter as its colloidal suspension in water. The effect on the gelatin was to produce a turbidity in the strip immersed in the solution under examination. Throughout the experiments the weight of protein, volume of solution, and temperature (20°) were kept constant. Observations of the effects of the various solutions upon the proteins were made immediately after admixture and also after standing one hour. The results tabulated below are based on the observations made after the one hour's standing.

The experimental results. The protein-precipitating powers of the various substances have been compared with their germicidal activities, which are expressed in the table below in terms of the concentrations required to disinfect *B. typhosus* or *Staphylococcus pyogenes aureus* in 15 minutes under the standard conditions employed by Chick and Martin [1908]. The concentrations have been selected from two previous papers on disinfection (1) by Morgan and Cooper [1912] and (2) by the author [1912, 1], to which papers the numbers 1 and 2 in the following table (p. 177) refer.

The following parallelisms can be deduced from these results.

1. The germicidal and protein-precipitating powers of phenol are decreased by the introduction of hydroxyl-groups into its benzene nucleus and increased by the introduction of nitro-groups and also by that of a methyl group.

2. The monohydric phenols are superior to the monohydric alcohols and also to acetone both as germicides and protein-precipitants.

3. The effects of substitution in the molecule of phenol upon its germicidal and protein-precipitating powers are frequently of somewhat similar orders of magnitude. An exact correspondence was not expected, firstly, because it is well known that the carbolic acid coefficient of a

substance varies to some extent with the species of micro-organism employed, and, secondly, because bacterial proteins were not used in the determination of protein-precipitating power.

TABLE I.

Substance	*Protein-precipitating power* Minimum concns. at which precipitation was visible. (Percentages in terms of weight per volume)	*Germicidal power* Concentrations killing in 15 minutes. (Percentages in terms of weight per volume)	*Organism*
	Gelatin 0·1 g.	Vol. of liquid 10 c.c.	
Phenol ...	2·5	0·8 [1]	*B. typhosus.*
Trinitrophenol (Picric acid)	0·2	0·1 [1]	,,
Resorcinol ...	4·0	2·6 [2]	,,
Catechol ...	5·0	1·7 [2]	,,
Quinol ...	5·0	0·75 [2]	,,
Pyrogallol ...	3·0	1·0 [2]	,,
Phloroglucinol	Satd. solns. (5 %) had no visible effect	>2·3 [2]	,,
p-Nitrophenol	0·9	0·46 [1]	*Staphylococcus py. aur.*
m-Nitrophenol	0·6	0·27 [1]	,, ,,
Trinitrophenol (Picric acid)	0·2	0·14 [1]	,, ,,
Ethyl alcohol	50 % and 100 % had no visible effect	35·0 [1]	,, ,,
Acetone ...		>10·6 [2]	,, ,,
Phenol ...	2·5	1·0 [1]	,, ,,
Egg-albumin ½ c.c. 10 % solution (0·05 g.).		Vol. of solution containing precipitant = 4·5 c.c.	
Phenol ...	1·0	0·8 [1]	*B. typhosus.*
		1·0 [1]	*Staphylococcus py. aur.*
m-Cresol ...	0·7	0·5 [2]	,, ,,
		0·3 [2]	*B. typhosus.*
Acetone ...	12·0	>10·6 [2]	*Staphylococcus py. aur.*
Methyl alcohol	20·0	35·0 [1]	,, ,,
Ethyl alcohol	20·0	35·0 [1]	,, ,,
		32·5 [1]	*B. typhosus.*
Propyl alcohol	12·0	14·0 [1]	*Staphylococcus py. aur.*

II. The Effects of Various Substances upon the Germicidal and Protein-Precipitating Action of the Phenols.

The experimental method employed in the investigation of the precipitating action of the phenols was similar to that described in Section I. The various substances were added to the phenol solutions immediately before admixture with the protein. The results recorded in this section are based on observations of the condition of the proteins after one hour's contact with the phenol solutions.

A. Sodium Chloride.

Reichel [1909] showed that the addition of sodium chloride increased both the bactericidal action of phenol and its solubility in proteins. The germicidal power was about doubled by 10 per cent. salt and quadrupled by 20 per cent. salt, and the effects of salt upon the solubility in proteins were of similar orders of magnitude. Smaller amounts of salt had little influence.

These experiments indicated how in the presence of salt a larger amount of phenol became available for disinfection, but did not throw any light on the mechanism of the germicidal action of this disinfectant. It was therefore of interest to investigate the influence of salt upon the protein-precipitating action of phenol.

Gelatin. The minimum concentrations of phenol, which induced a visible precipitation in the presence of varying amounts of sodium chloride, were determined. The results are tabulated below:

TABLE II.

Percentage of salt present (weight per volume)	Minimum phenol concentrations precipitating the protein
0	3 %
5	2·5
12	1·5
19	0·75

Although saturated aqueous solutions of *m*-cresol (1·25 per cent.) had no visible effects upon gelatin, this protein was precipitated by 0·8 per cent. solutions of *m*-cresol in the presence of 9 per cent. salt and even by 0·4 per cent. solutions in the presence of 20 per cent. of salt.

Dialysed egg-albumin. 3·3 per cent. aqueous solutions of phenol only induced a faint turbidity in a sample of egg-albumin, but in the presence of 6 per cent. salt an extensive precipitation occurred. 1 per cent. solutions of phenol in water and in 6 per cent. salt had no visible effect on the albumin suspension; a turbidity however was induced by the same phenol concentration in 15 per cent. salt, and a marked precipitation in 22 per cent. salt solution.

The precipitating action of phenol upon proteins was thus greatly increased by the presence of salt, but was not appreciably affected until high salt concentrations were attained.

A comparison of the results of experiments with gelatin with those obtained by Reichel [1909] indicates that the germicidal power, the solubility in proteins, and the precipitating action of phenol are increased to very similar extents by the presence of salt.

The increased germicidal power thus appears to be due to an increased de-emulsifying action of the phenol upon the bacterial proteins resulting from the availability within the emulsoid particles of a larger amount of phenol.

B. Alcohol.

Kronig and Paul [1897] showed that the germicidal action of phenol upon spores was decreased by the presence of alcohol and that solutions of phenol in 98 per cent. alcohol possessed no bactericidal power.

The author [1912, 2] found that the uptake of phenol by a protein was greatly decreased by the presence of alcohol, the protein absorbing for example eight times as much phenol from aqueous solution as from a solution of phenol in 50 per cent. alcohol. These experiments indicated how in the presence of alcohol less phenol became available for disinfection, but they afforded no explanation of the nature of its germicidal action. It was therefore of some importance to investigate the influence of alcohol upon the protein-precipitating action of phenol.

Gelatin in the form of strips was chiefly employed in the experiments, because it was not precipitated by immersion in alcohol of any strength. Although this protein was precipitated by $2\frac{1}{2}$ per cent. aqueous phenol solutions, there was no indication of precipitation by 5 per cent. phenol solutions in 50 per cent. alcohol, and the extent of the precipitation by 6·25 per cent. phenol solutions in 25 per cent. alcohol was much less than that induced by the above aqueous solutions. Furthermore, 10 per cent. solutions of phenol in absolute alcohol had no visible effect upon gelatin. This is of interest when correlated with Kronig and Paul's observation that solutions of phenol in 98 per cent. alcohol possessed no germicidal power.

A sample of dialysed egg-albumin was immediately precipitated by 1·5 per cent. aqueous phenol solutions, but was not visibly affected by the same concentration in the presence of 10 per cent. alcohol.

Although 0·5 per cent. aqueous solutions of picric acid precipitated gelatin, similar concentrations in 30 per cent. alcohol had no visible effect upon this protein.

The inhibiting effect of alcohol upon the germicidal power of phenol thus appears to be due to a decrease in the de-emulsifying action of the latter upon the bacterial proteins, resulting from a diminution in the amount of phenol available within the emulsoid particles.

C. Fat.

Strips of gelatin were immersed in castor-oil, containing varying amounts of phenol. It was found that solutions of phenol in the fat as strong as 10 per cent. (by weight) had no visible precipitating action upon gelatin, although this protein was precipitated by $2\frac{1}{2}$ per cent. aqueous solutions of phenol.

This can be correlated with the fact discovered by Koch that solutions of carbolic acid in oil possess no germicidal power. The greatly diminished protein-precipitating action of phenol in the presence of fat is probably due, as in the case of alcohol, to a decreased uptake of phenol by the protein, because, while phenol is about three times as soluble in gelatin as in water, the partition-coefficient for phenol between fat and water is six.

D. Alkali.

Kronig and Paul [1897] showed that, while 4 per cent. solutions of phenol in water exerted a strong germicidal action upon anthrax spores, solutions of sodium phenate equivalent to 4 per cent. phenol possessed no germicidal power.

Morgan and Cooper [1912] showed that *p*-nitrophenol was about five times as powerful a germicide as its potassium salt. The inhibiting action of alkali upon the agglutination of proteins by heat suggested that the decreased germicidal power might be due to the protective effect upon the bacterial protoplasm of the alkali, produced in the hydrolysis of the alkali phenates, against the precipitating action of the phenols simultaneously liberated. Accordingly the effect of alkali on the protein-precipitating action of the phenols has been studied.

Dialysed serum-albumin: Phenol. The albumin was not visibly affected by 0·9 per cent. solutions of phenol, but was precipitated by 1·7 per cent. phenol. The precipitation was not only completely inhibited by N/50 to N/150 concentrations of caustic soda, but even by a concentration as low as N/300.

Gelatin: Phenol. Although gelatin was precipitated by 2·5 per cent. aqueous phenol solutions, this protein was not visibly affected by 4 per cent. phenol in the presence of N/25 caustic soda and was only very slightly precipitated by the same concentration in presence of N/50 caustic soda.

Traces of organic bases, such as ethylamine and ethylenediamine, also inhibited the precipitation of gelatin and egg-albumin by phenol.

Gelatin: p-Nitrophenol. Gelatin was precipitated by 1 per cent. aqueous solutions of *p*-nitrophenol, but was not visibly affected by the same concentration in the presence of N/100 caustic soda, nor even by 1·4 per cent. (N/10) solutions containing N/10 caustic soda.

Not only could alkali inhibit the precipitation of proteins by the phenols, but when present in concentrations varying from N/1 to N/10 it could disperse egg-albumin already precipitated by phenol.

The results indicate that very low concentrations of alkali are sufficient to inhibit the precipitation of proteins by phenol. As alkali combines with the phenols forming salts, which are partially hydrolysed in solution, the inhibiting concentrations must have been even smaller than those stated above.

In view of these results it was of great interest to investigate the influence of alkali upon the germicidal action of phenol in greater detail, and in particular to study the effects of very low alkali concentrations. The experiments consisted in the estimation of the minimum concentrations of phenol required to disinfect in a specified time under standard conditions in the presence of varying amounts of alkali.

The following results were obtained:

TABLE III.

Organism, B. typhosus.

Concentration of caustic soda present	Minimum concentration of phenol required to disinfect in 15 mins. at 20°
0	9·5 in 1000 (N/10)
N/250	,, ,,
N/50	,, ,,
N/25	11 in 1000 (N/8·5)
N/20	,, ,,
N/15	13·5 in 1000 (N/7)

The germicidal powers of the above concentrations of alkali in the absence of phenol were next determined and are compared in the following table with those of solutions containing initially the same alkali concentrations with varying amounts of phenol.

TABLE IV.

Concentration of NaOH		Time required for complete disinfection
N/500 NaOH		> 5½ hours
N/250 ,,		> 5½ ,,
N/100 ,,		45 minutes
N/50 ,,		20 ,,
N/25 ,,		< 3 ,,
N/15 ,,		< 3 ,,
N/10 ,,		< 3 ,,
N/25 ,,	+ N/10 phenol	> 15 ,,
N/25 ,,	+ N/8·5 ,,	15 ,,
N/20 ,,	+ N/10 ,,	> 15 ,,
N/20 ,,	+ N/8·5 ,,	15 ,,
N/15 ,,	+ N/10 ,,	> 15 ,,
N/15 ,,	+ N/8·5 ,,	> 15 ,,
N/15 ,,	+ N/7 ,,	15 ,,
N/10 ,,	+ N/12 ,,	< 15 ,,
N/10 ,,	+ N/10 ,,	15 ,,
N/10 ,,	+ N/9 ,,	> 15 ,,
N/10 ,,	+ N/7 ,,	> 15 ,,

The above results indicate that alkali decreased the germicidal power of phenol when present in moderately low concentration. While, however, the precipitation of serum-albumin by phenol was inhibited by an initial concentration of N/300 caustic soda and that of gelatin by N/50 caustic soda, bactericidal power was not measurably affected until the initial alkali concentration was raised to N/25. The precipitating action of phenol was therefore much more sensitive to the inhibiting effect of alkali than its germicidal action. This discrepancy is not sufficiently explained by the slight acidity of the broth culture, nor by the toxic action of the alkali, since an analysis of the results tabulated above indicates that not only did small concentrations of alkali such as N/250 exert a very feeble germicidal action, but furthermore the bactericidal action of the alkali was greatly decreased by the presence of phenol. Owing to this depreciating effect N/10 solutions of alkali, containing initially N/7 or N/9 concentrations of phenol, possessed a smaller germicidal power than those which initially contained lower phenol concentrations such as N/10 and N/12.

It is possible that a larger amount of alkali is required to preserve the colloidal suspension from the action of the phenol in the case of the bacterial proteins than in the cases of gelatin and albumin.

E. Acids.

Hailer [1910] showed that acids, arranged according to their germicidal power and also according to their efficiencies in increasing the bactericidal power of phenols, were not in the same order. Thus sulphuric acid exceeded oxalic acid in bactericidal power, but was less effective in increasing the germicidal power of phenol. Some of the experimental results described in the previous pages suggested that the discrepancy might be due to differences in the effects of the acids upon the protein-precipitating action of phenol. This, however, seems not to be the case because it was found that, although acids greatly increased the precipitating action of phenol, sulphuric acid was effective in much lower concentration than oxalic acid.

The effects of alcohol and salt upon the precipitating action were shown to be due to a redistribution of phenol between the water and proteins. The greatly increased precipitating action in the presence of acids however is not sufficiently explained in this way, as it was previously shown [Cooper, 1912, 2] that the addition of acid only very slightly increased the solubility of phenol in protein. It is probably due to the formation of an acid salt of the protein, which is more readily precipitated than the protein itself.

Discussion of Results.

The parallelisms deduced from the study of the effects of changes in the chemical constitution of phenol and of the presence of certain substances (e.g. salt, alcohol, fat) upon its germicidal and protein-precipitating powers support the conclusion arrived at in the previous communication, namely, that the germicidal action of phenol is associated with an alteration in the condition of the colloidal suspensions of the bacterial proteins as evidenced by the precipitation of such proteins as gelatin and albumin when certain phenol concentrations are attained.

It has already been pointed out that the heat coagulation of proteins consists of two stages.

1. A reaction between the proteins and hot water (denaturation).
2. A separation of the protein in a particulate form (agglutination).

It is therefore of interest to attempt to characterise the essential process in disinfection by the phenols as either a denaturation of the bacterial proteins or a de-emulsification of their colloidal solution. Chick and Martin [1911; 1912] have shown that the process of denaturation by heat

is accelerated by acid and alkali, and retarded by salt, while the subsequent process of agglutination is facilitated by low concentrations of acid and salt, but inhibited by high concentrations and also by alkali.

It was shown in Section II of this paper that the bactericidal power of phenol, its protein-precipitating action and solubility in proteins were increased to equal extents by the addition of salt. The latter thus appears to have no *direct* effect upon the germicidal and precipitating actions of phenol, but to increase them merely by raising the phenol concentration within the emulsoid protein-particles. It is therefore not possible by a study of the effects of salt to correlate these properties of phenol with either the denaturating or agglutinating action of heat upon proteins.

The fact, however, that the germicidal action of phenol is inhibited by low concentrations of alkali, although to a less extent than its precipitating action upon proteins, suggests that the essential process in disinfection by phenol resembles the final phase in the heat-coagulation of proteins and consists in the destruction of the colloidal suspensions of the bacterial proteins.

Since the process of disinfection by hot water was found [Chick, 1910] to be accelerated by alkali as well as by acid, it is possible that the essential stage in this process is the denaturation of the bacterial proteins by the action of the water and not the subsequent precipitation. A study of the effects of salt upon the velocity of disinfection by hot water will throw light on this problem.

Summary.

1. The germicidal and protein-precipitating powers of phenol are similarly affected by the entrance of various chemical groups into its molecule.

(*a*) The introduction of hydroxyl groups decreases and that of nitro-groups and of a methyl group increases the bactericidal and protein-precipitating powers of phenol.

(*b*) The monohydric phenols are superior to the alcohols both as germicides and protein-precipitants.

2. Sodium chloride increases the germicidal and protein-precipitating action of phenol through increasing its solubility in proteins.

3. Alcohol, on the other hand, decreases the germicidal and protein-precipitating action of phenol through diminishing its solubility in proteins. Solutions of phenol in absolute alcohol exert no germicidal action upon spores and have no visible precipitating action on gelatin.

4. Solutions of phenol in fat also possess no germicidal power and do not visibly precipitate gelatin.

5. While the presence of very small amounts of alkali is sufficient to inhibit the precipitating action of phenol upon proteins, only moderately low alkali concentrations can effect a measurable decrease in its germicidal power. The explanation of this discrepancy is not apparent.

6. The precipitating action of phenol is increased by the addition of acids.

Main Conclusion.

From the evidence set forth in the previous communication and in the present paper it is concluded that the absorption of phenols by bacteria is merely the initial stage in the process of disinfection, and that the germicidal action which follows is due, not to a typical chemical union of the phenols with the bacterial protoplasm, as appears to be the case with formaldehyde, but to a de-emulsifying action upon the colloidal suspension of some constituent protein or proteins essential for the vitality of the organisms.

I desire to express my best thanks to Prof. Martin, F.R.S., for much helpful criticism of this work.

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XVII. ON THE RELATIONS OF THE PHENOLS AND THEIR DERIVATIVES TO PROTEINS. A CONTRIBUTION TO OUR KNOWLEDGE OF THE MECHANISM OF DISINFECTION.

PART III. THE CHEMICAL ACTION OF QUINONE UPON PROTEINS.

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(*Received January 13th, 1913.*)

Blyth and Goodban [1907] found that when pure cresylic acid was exposed to light and air until it had become brown its germicidal power was measurably increased. This was probably due to the formation of derivatives of quinone through the aerial oxidation of the cresols.

Thalhimer and Palmer [1911] however could not detect any increase in germicidal power when phenol was exposed to light and air for some time.

Morgan and Cooper [1912] showed that some of the aromatic amines possessed a higher germicidal power when coloured by long standing than when purified by redistillation. It is probable that the increased germicidal power was due to the production of coloured quinone derivatives, which are known to be formed in the oxidation of aromatic amines.

Thalhimer and Palmer [1911] showed that quinone itself was a very efficient disinfectant and was superior to phenol, cresol, quinol, phenoquinone, and formalin in germicidal power.

The author [1912, 1] confirmed some of the latter results and showed further that quinone was more efficient as a germicide than the aliphatic ketone, acetone.

In previous communications [Cooper, 1912, 2; 1913] evidence was set forth which strongly suggested that the germicidal action of the phenols was due, not to a typical chemical union with the bacterial proteins, but to a de-emulsifying effect upon their colloidal suspensions. In view of the high bactericidal power of quinone it was of great interest to investigate the

relations of this ketone to proteins, in order to compare the mechanism of its germicidal action with that of the phenols and to arrive at a conclusion as to the possibility of a relationship between its germicidal efficiency and chemical reactivity.

Würster [1889] showed that when quinone was added to warm solutions of various amino-acids a red coloration was developed.

Raciborski [1907] showed that quinone gave a red coloration not only with amino-acids, but also with peptone and several proteins (egg-albumin, serum-albumin, fibrin, globulin, legumin, and nuclein). Toluquinone reacted similarly to quinone, and xyloquinone also gave a colour-reaction with proteins and peptone, but not with glycine and alanine. Phenanthraquinone and anthraquinone, on the other hand, gave no colour reactions with proteins. The authors put forward no explanation of the above phenomena.

The Experimental Results.

The investigation described in this paper is divided into three parts.

Part I deals with the relations of quinone to various proteins.

Part II deals with the nature of the chemical action, which quinone was found in Part I to exert upon proteins.

Part III deals with the relation of the chemical reactivity of quinone to its germicidal power.

I. *The relations of quinone to various proteins.*

Gelatin.

When strips of gelatin were immersed in aqueous solutions of quinone (0·1 per cent.) the protein rapidly developed an intense red colour, but retained its transparency. The reaction was irreversible, as the colour was not removed by prolonged boiling with water or absolute alcohol. The red colour was changed to green by the addition of alkali, but was restored by acidification. The altered gelatin was, furthermore, completely insoluble in hot water, and after immersion for 16 hours in quinone solutions the protein was no longer rendered opaque (precipitated) by phenol. Immersion for 30 minutes did not inhibit the precipitation.

Gelatin after immersion in 40 per cent. formalin for 12 hours was also insoluble in hot water and was no longer precipitated by 5 per cent. phenol, although the original gelatin was visibly affected by 2·5 per cent. phenol solutions.

These facts suggest that the quinone was not merely dissolved by the

gelatin, but had reacted chemically with the protein. The colour-reaction did not occur when the gelatin was immersed in solutions of quinone in absolute alcohol, and it only took place to a very small extent in 20 per cent. alcohol. Solutions of quinone in toluene also gave no coloration with gelatin.

Similarly, although Witte's peptone was coloured intensely red when suspended or dissolved in aqueous quinone solutions, no coloration was observed when it was suspended in an alcoholic solution of this substance.

The interpretation of these facts is probably that the quinone is *dissolved* in the gelatin and proteoses before the chemical reaction, so that an efficient external solvent for quinone (such as alcohol) by decreasing the uptake of this substance by the colloids can inhibit the colour-reaction. (See the effect of alcohol upon the distribution of phenol between water and proteins, Cooper [1912, 2].)

Alizarin, like anthraquinone, gave no coloration with proteins. When however gelatin was immersed in strong aqueous solutions of sodium alizarin-sulphonate (Alizarin red, $C_{14}H_5O_2(OH)_2SO_3Na$) it assumed an intense red colour, but, unlike gelatin treated with quinone, it was still readily soluble in hot water and precipitated by 5 per cent. phenol, and the colour was rapidly removed by washing with cold water.

Alizarin and its sulphonic derivative thus differed from quinone in not reacting with proteins, and this inactivity may explain the fact that a saturated solution (0·2 per cent.) of alizarin and a 1 per cent. solution of alizarin-red exerted no measurable germicidal action upon Staphylococcus py. aur.

Caseinogen.

(Merck's Casein—prepared according to Hammarsten.)

When immersed in aqueous solutions of quinone this protein assumed a purple colour, which was not removed by washing with water or boiling alcohol. Unlike the original caseinogen the coloured product (after being washed with water only) was insoluble in N/5 soda. Contact with the alkali, however, turned it green, but the purple colour was restored by acidification. The altered caseinogen was very slowly soluble in hot concentrated hydrochloric acid yielding a brown solution, and thus again differed from the original protein, which quickly dissolved in this acid forming a violet solution.

Egg-albumin.

As stated by Raciborski [1907] dialysed egg-albumin when mixed with aqueous solutions of quinone soon gave an intense red coloration. The

protein was still coagulated by heat and alcohol, red coagula being formed, which were not decolorised by prolonged washing with water and alcohol. The protein was also precipitated from the red solution by saturated ammonium sulphate. The precipitate was a red flocculent substance, readily soluble in water and becoming dark brown on standing. After this colour change the protein was frequently found to have become almost insoluble in water. The coagula obtained from the original red solution by means of heat, alcohol, and phenol remained permanently red.

Horse-Serum.

When aqueous solutions of quinone were added to horse-serum a red coloration rapidly developed. The serum-proteins were still coagulated by heat and precipitated by alcohol at first reversibly and finally irreversibly. The coagula were red and could not be decolorised by prolonged washing with alcohol or water.

By half-saturation of the red solution with ammonium sulphate a red flocculent precipitate was obtained, which, like the egg-albumin precipitated by ammonium sulphate from solutions containing quinone, became dark brown on standing. The precipitate with magnesium sulphate and the alcohol coagulum, on the other hand, resembled the egg-albumin coagula in remaining permanently red, but they soon became brown when immersed in a solution containing ammonium sulphate and quinone. The cause of these colour-changes could not be discovered.

By acidifying with acetic acid the filtrate from the precipitation with half-saturated ammonium sulphate of the serum containing quinone another red precipitate was obtained, corresponding to the albumin fraction from normal serum. The precipitate quickly redissolved on the addition of water.

It was not found possible to crystallize the quinone derivates of serum and egg-albumin by the application of the usual methods.

Witte's Peptone.

The observation of Raciborski [1907] that solutions of Witte's peptone gave a red coloration with quinone was confirmed. Experiments were next carried out with a view to the isolation of the coloured products.

Witte's peptone has been separated into five constituents by the method of fractional precipitation with alcohol and salt described by Haslam. The following fractions of proteoses have been isolated in this way:

1. Insoluble in 50 per cent. alcohol and water. Crude hetero-proteose.
2. Insoluble in 50 per cent. alcohol and soluble in water.

(1) *a*-proto-proteoses—precipitated by half-saturated $(NH_4)_2SO_4$.

(2) *a*-deutero-proteoses—precipitated by saturated $(NH_4)_2SO_4$.

3. Soluble in 50 per cent. alcohol and soluble in water.

(1) β-proto-proteoses—precipitated by half-saturated $(NH_4)_2SO_4$.

(2) β-deutero-proteoses—precipitated by saturated $(NH_4)_2SO_4$.

By similarly fractionating the coloured liquid obtained by mixing solutions of quinone and Witte's peptone together it was possible to isolate corresponding precipitates all of which were highly coloured. Some of these products, however, did not differ merely in colour from the fractionated proteoses. Thus, while the alcoholic precipitate (*a*-proteoses and hetero-proteoses) obtained from an aqueous solution of Witte's peptone was readily soluble in warm anhydrous *m*-cresol, the corresponding fraction obtained from the aqueous solution after treatment with quinone was only slightly soluble.

Again, the *a*-proteoses after reacting with quinone were no longer precipitated by formaldehyde, although they were still precipitated by alcohol, mercuric chloride, and phosphotungstic acid. The significance of this difference is discussed later. The fractions obtained from Witte's peptone after treatment with quinone corresponding to the *a*- and β- proteoses gave the characteristic test for proteoses, namely a precipitate with nitric acid, soluble on warming and reappearing on cooling.

The main conclusion to be drawn from these results is that proteins isolated after treatment with quinone are permanently coloured and frequently changed in solubility and precipitability, and thus appear to be chemically altered. In the following pages the nature of this chemical change and the possibility of its relationship to the toxic action of quinone upon bacteria are discussed.

II. *The nature of the chemical action of quinone upon proteins.*

(i) *The colour reactions given by quinone with simple amines.*

A large number of amines readily gave colorations with quinone in aqueous solution at ordinary temperatures.

(ii) *The colour-reactions with imino-compounds.*

In cold aqueous solution quinone gave red colorations with methylaniline and di-amylamine, but not even on warming with succinimide, acetanilide, and uric acid. Under certain conditions quinone could thus react with substances containing the $=NH$ group.

(iii) *The inhibitory effect of formaldehyde upon the colour-reactions.*

The fact that quinone gives colorations with amino- and imino- compounds

suggests that the colour-reaction with proteins is due to a condensation of the ketonic groups of the quinone with their $-NH_2$ and $=NH$ groups.

Formaldehyde is known to react with amino- and imino- compounds by condensation with the $-NH_2$ and $=NH$ groups, forming methylene derivatives.

$$NH_2 \cdot CH_2 \cdot COOH + H \cdot CHO = CH_2 : N \cdot CH_2 \cdot COOH + H_2O$$

$$2NH(CH_3) \cdot CH_2 \cdot COOH + H \cdot CHO = H_2C\begin{matrix} N(CH_3) \cdot CH_2 \cdot COOH \\ N(CH_3) \cdot CH_2 \cdot COOH \end{matrix} + H_2O$$

The colour reactions given by quinone with proteins, if due entirely to a similar condensation, should therefore be inhibited by the previous formalisation of these substances.

Substance	Nature of reaction
Ammonia	Brown coloration.
Ammonium sulphate ...	Purple ,,
Ethylamine	Violet ,,
Di-amylamine	Rose red ,,
Guanidine (carbonate) ...	Intense green coloration. (Hot solutions brown.)
Glucosamine	Red coloration.
Amino-antipyrine	Purple ,,
Tryptophane	Red ,,
Atoxyl	Red ,,
Aniline	Red ,, (followed by brown precipitate).
o-Phenylene-diamine ...	
m-Tolylene-diamine ...	Violet ,, ,, ,, ,,
β-Naphthylamine	Brown ,,
Tetra-hydro-*β*-naphthylamine	Brown oil.
Pyridine	Dark yellow coloration passing to red.
Succinanilamine	Red coloration (followed by red precipitate).

It was actually found that in the case of egg-albumin, serum-proteins proteoses, glycocoll, glycyl-*l*-tyrosine, methylaniline, and di-amylamine the colour reactions with quinone were entirely inhibited by adding 40 per cent. formalin to the compounds either previously to or simultaneously with the addition of the quinone. Smaller amounts of formaldehyde (one to ten per cent.) added with the quinone were found to inhibit the colour-reaction partially.

Conversely, the *a*-proteoses present in Witte's peptone after treatment with quinone were no longer precipitated by formaldehyde, although they were still precipitable by alcohol, phosphotungstic acid, and mercuric chloride.

In the case of gelatin, however, formalisation did not inhibit the colour-reaction with quinone. This was also true in the case of ammonia, and here the absence of any inhibiting effect could not be due to incomplete interaction

with formaldehyde, because the product of this reaction—hexamethylene-tetramine (urotropin), which was proved to be ammonia-free, readily gave a red coloration with quinone.

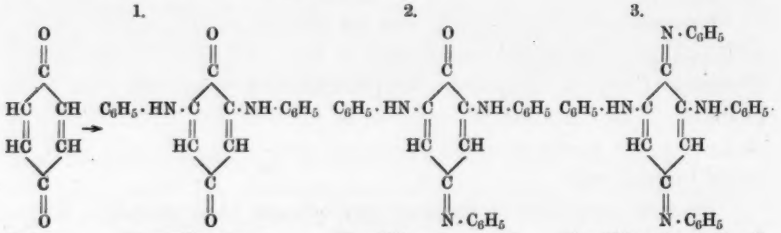
The purified product from the interaction of formaldehyde and aniline also gave a colour-reaction with quinone.

The inhibiting effect of formalin upon the colour-reaction given by quinone with certain proteins and other amino- and imino- compounds, and its inability to precipitate the compounds of quinone with α-proteoses, although it readily precipitates the proteoses themselves, confirm the view that it is the $-NH_2$ and $=NH$ groups present in the proteins and their hydrolytic products that react with the quinone. It is difficult to understand, however, why the products of formalisation of gelatin, ammonia, and aniline should behave exceptionally in yielding a coloration with quinone.

In order to attempt to understand the mechanism of the interaction of quinone and proteins it is necessary here to set forth the course of the reaction known to occur between an amine, such as aniline, and the above ketone.

The products of the reaction depend upon the experimental conditions, as indicated below.

1. As a result of the action of aniline upon an alcoholic solution of quinone there are three products:



2. In the presence of acetic acid the chief product is di-anilido-quinone-anil.

3. By fusing quinone with aniline and its hydrochloride the chief product is di-anilido-quinone-di-anil.

There are thus two possible reactions between quinone and proteins and their hydrolytic products:

1. The condensation of the ketonic oxygen atoms with the hydrogen of the amino- and imino-groups, forming compounds of the anil-type (see above 2, 3).

2. The replacement of hydrogen attached to the benzene nucleus of quinone by amino-acid residues through the amino- or imino-groups forming compounds of the anilido-type (see above 1, 2, 3).

These two reactions might proceed simultaneously forming compounds analogous to di-anilido-quinone-di-anil.

(iv) *Experiments with quinone-dioxime.*

Attempts were next made to discover to which type of chemical reaction the red colorations given by quinone with proteins were due.

It was thought that experiments with quinone-dioxime could decide this question, because, although substitution in the ketonic groups naturally prevents their condensation with amino- and imino-compounds, it is known not to inhibit the entrance of these substances through their nitrogen atoms into other parts of the quinone nucleus.

Quinone-dioxime was prepared by the action of hydroxylamine hydrochloride upon quinone, the reaction being carried out in acid solution to prevent the reduction of the quinone to quinol.

It was found that aqueous solutions of quinone-dioxime gave no colorations with serum, gelatin, proteoses and alanine. The gelatin after immersion in the quinone-dioxime solution was found to be still soluble in hot water and precipitated by phenol as a white substance, and was thus not chemically altered.

These results strongly suggest that the colour-reaction given by proteins with quinone is due to the condensation of the $-NH_2$ or $=NH$ groups with the ketonic groups of the quinone, compounds similar to quinone-dianil being produced. This conclusion is supported by the fact that no oxime could be obtained by the treatment of the quinone-proteose compounds with hydroxylamine hydrochloride.

The chemical action of quinone upon proteins thus resembles that of formaldehyde.

(v) *The relations of acetone to proteins.*

Since it was found that quinone possessed a germicidal power more than 100 times as great as that of acetone [Cooper, 1912, 1] it was of interest to compare the effects of these two ketones on proteins.

Acetone was found to differ from quinone in exerting a precipitating action upon proteins, but while 0·1 per cent. solutions of quinone gave a colour-reaction with egg-albumin and gelatin, the albumin was not precipitated by aqueous solutions of acetone below 12 per cent. and gelatin was not even affected by immersion in 50 per cent. and 90 per cent. solutions.

The gelatin was still soluble in hot water and precipitable by phenol after this treatment, so that there was no evidence that it was chemically altered by the acetone. This ketone is therefore to be classed with the alcohols and phenols as a protein-precipitant.

III. *The relation of the chemical reactivity of quinone towards proteins to its germicidal power.*

The fact that as a germicide quinone is greatly superior to many other para-di-substitution products of benzene is seen from the following table [Cooper, 1912, 1 and Morgan and Cooper, 1912].

Substance	Organism	Carbolic coefficient
Quinone	*Staphylococcus py. aur.*	10
Benzaldehyde ...	*B. typhosus*	10
Quinol	,,	1
p Cresol	*Staphylococcus py. aur.*	2·4
,, ...	*B. typhosus*	2·6
Aniline	*Staphylococcus py. aur.*	0·5
,, ...	*B. typhosus*	0·57
p-Toluidine ...	,,	1·25
p-Phenylene-diamine	,,	Under 0·3
p-Nitrophenol ...	*Staphylococcus py. aur.*	2·3

This itself suggests that the high germicidal power of quinone is associated with its chemical reactivity.

This conclusion is supported by certain other facts.

1. Not only is quinone superior to phenol, *p*-cresol, quinol, *p*-nitrophenol and acetone in germicidal power, but it can exert a chemical action upon proteins in concentrations (e.g. 0·1 per cent.) much lower than those in which the above substances can induce protein precipitation [Cooper, 1912, 2; 1913].

2. Benzaldehyde, which resembles quinone in its chemical action upon proteins, is also approximately equal to quinone in germicidal power, its carbolic coefficient with *B. typhosus* being 10.

There is thus some evidence that the mechanism of the germicidal action of quinone consists in a chemical interaction with some constituent protein or proteins of the bacteria essential for vitality, and not, as seems to be the case with the phenols, in a precipitating effect upon the colloidal suspension. The superiority of quinone as a germicide to various phenols and to acetone is sufficiently explained by the fact that it reacts with proteins in concentrations much lower than those in which the phenols and acetone exert a precipitating action.

Summary.

1. The observations of Würster and Raciborski that quinone solutions gave a red coloration with various proteins and amino-acids have been confirmed.

2. The proteins (egg-albumin, proteins of horse serum, gelatin, Witte's peptone) could be isolated in the coloured condition from the red solutions by means of various precipitants and could not be decolorised by prolonged washing with water or alcohol. Other physical properties of the proteins, e.g. solubility, precipitability, were frequently changed as a result of the treatment with quinone, as is also the case when proteins react with formaldehyde. From these results it appeared that the proteins had become chemically altered by the quinone.

3. The colour-reaction did not occur when gelatin and proteoses were immersed in solutions of quinone in absolute alcohol. It would appear that the quinone was dissolved by the colloids before the chemical reaction, so that an efficient solvent for this ketone such as alcohol, by decreasing the uptake, could inhibit the colour-reaction.

4. The addition of sufficient formaldehyde to proteins, proteoses, amino-acids, and imino-compounds either before or simultaneously with the addition of the quinone completely inhibited the colour-reactions. Smaller amounts of formalin decreased the intensity of the red colorations. Gelatin, aniline, and ammonia however behaved exceptionally inasmuch as they still gave the colorations with the quinone after the addition of formalin. The positive results appeared not to be due to incomplete formalisation, since the isolated compounds of aniline and ammonia with formalin gave colour-reactions with quinone.

The inhibitory effect of formalin upon the colour-reactions given by quinone with certain proteins, with proteoses and amino-acids indicates that the latter react with quinone through their $-NH_2$ or $=NH$ groups.

5. Proteins, proteoses, and alanine gave no colour-reaction with quinone-dioxime, and no oxime could be prepared from the quinone-proteose compounds. This is presumptive evidence that the constituent $-NH_2$ or $=NH$ groups of the proteins and their hydrolytic products condense with the ketonic groups of the quinone. The chemical action of the latter upon proteins thus resembles that of formaldehyde.

6. Acetone differed from quinone in acting as a protein-precipitant.

7. There is some evidence that the germicidal power of quinone is due

to its chemical action upon some constituent protein or proteins of the bacterium essential for vitality and that the superiority of quinone as a germicide to phenol, quinol, and acetone is explained by its reactivity towards proteins in much lower concentration.

I desire to express my best thanks to Prof. Martin, F.R.S., for helpful criticisms of this work.

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XVIII. THE RATE OF FERMENTATION BY GROWING YEAST CELLS.

By ARTHUR SLATOR.

(*Received January 16th, 1913.*)

If a sugar solution which also contains the necessary food for yeast growth is seeded with a small quantity of yeast, the yeast grows and the sugar is fermented to alcohol and carbon dioxide. If the seeding is small and all necessary food for yeast growth is in excess the growth during the earlier stages of the reaction is unrestricted and follows the logarithmic law of increase, that is the rate of increase is always proportional to the quantity present.

At a later period retarding influences come into action, the yeast multiplication becomes restricted and during the final stages of the fermentation ceases entirely.

In this communication an account is given of some measurements made during the earlier part of the reaction where the yeast growth is unrestricted.

If certain simple assumptions regarding fermentation are made, it can be shown that during this period not only does yeast growth, but also the fermentation caused by the yeast, follow the logarithmic law.

If the medium is seeded with N cells per c.c. then the rate of growth at any time t is proportional to the number of cells present $N+n$, where n is the increase during the time t, that is,

$$\frac{dN}{dt} = K(N+n) \quad \text{.....(1).}$$

where K is the constant of growth.

On integration this becomes

$$K = \frac{1}{t} ln \frac{N+n}{N} \quad \text{.....(2),}$$

or

$$n = N(e^{Kt} - 1) \quad \text{.....(2 a).}$$

If F represents the number of grams of sugar fermented per unit of time by each yeast cell and s the total amount fermented in time t, then s is determined by the equation

$$s = \int_0^t (N+n) F \, dt.$$

Substituting from equation 2 *a* we have

$$s = \int_0^t NF e^{Kt}\, dt$$

which on integration becomes

$$s = \frac{NF}{K}(e^{Kt} - 1) \quad \text{.....(3),}$$

or from equation 2 *a*

$$s = \frac{nF}{K} \quad \text{.....(4).}$$

If S is the amount of sugar fermented when the yeast grows from a very small seeding to N, then from equation 4

$$S = \frac{NF}{K} \quad \text{.....(5).}$$

Equation 3 therefore becomes

$$s = S(e^{Kt} - 1) \quad \text{.....(6)}$$

or

$$K = \frac{1}{t} ln \frac{S+s}{S} \quad \text{.....(6 a).}$$

It is evident from this equation that the time-fermentation curve during this period is logarithmic and that the constant of the curve is the constant of growth.

The validity of these equations has been tested by measuring the rate of growth of a pure culture of a Burton yeast in lightly hopped wort of specific gravity 1·040. It was found that K the constant of unrestricted growth of this culture could be determined by methods of yeast counting and also by measuring the rate of fermentation of the growing yeast cells.

Four different methods of estimating K have been worked out and when tested with this growth of yeast were found to give almost identical results.

In all these experiments the seeding was made with actively growing yeast cells so that any initial retardation in the growth which would take place if older yeast cells were used is eliminated.

Method 1.

The medium is seeded with a known number of yeast cells under sterile conditions. Tubes containing the inoculated solution are kept slowly agitated in a thermostat. At certain intervals of time the tubes are taken out and the number of yeast cells counted. K is calculated according to equation 2 which can be put in the form

$$0{\cdot}434\, K = \frac{1}{t} \log \frac{N+n}{N}.$$

TABLE I.

Temp. 20°.
t=time in hours.
$N+n$=number of yeast cells per c.c. at time t.

t	$N+n$	$0{\cdot}434\,K$
0	90,100	—
17·3	4,660,000	0·100
0	255,000	—
7·0	1,390,000	0·105
9·0	2,200,000	0·104
0	1,360	—
35	3,550,000	0·098
	Average	0·102

$T=2{\cdot}95$ hrs.

In the above table T is the time for the yeast to increase to twice the original amount and is determined by the equation

$$T=\frac{\log 2}{0{\cdot}434\,K}=\frac{0{\cdot}301}{0{\cdot}434\,K}.$$

METHOD 2.

Measurements of the rate of fermentation are carried out in the manner described in previous publications. [Slator, 1906, 1908.]

The sterile medium (75 c.c.) is seeded with a small seeding of yeast in a tube of capacity about 100 c.c. The tube is connected with a manometer to measure the rate of production of carbon dioxide. With a suitable seeding appreciable fermentation starts in about 15 hours, the reaction proceeds faster as the time goes on and measurements are continually taken on the manometer at suitable intervals.

The readings on the scale can be used directly to test equation 6 a

$$0{\cdot}434\,K=\frac{1}{t}\log\frac{S+s}{S}.$$

TABLE II.

Temp.=20°.
t=time in hours from first reading S.
$S+s$=manometer reading in cm., at time t taking the infinitely early reading as zero.

t	$S+s$	$0{\cdot}434\,K$
−15	(0·3)*	—
0	9·7 (S)	—
0·5	10·9	0·101
0·83	11·85	0·105
1·25	12·9	0·099
1·58	13·9	0·099
1·92	15·05	0·099
	Average	=0·101

A second experiment gave $0{\cdot}434\,K=0{\cdot}100$. $T=3{\cdot}0$ hrs.

* NOTE. 15 hrs. before the first reading is too short a time to give exactly the infinitely early reading and a small correction has to be made to obtain this value. As T is approximately 3 hrs. this correction is easily shown to be 1/32 of S, that is in this case 0·3 cm.

Method 3.

The constant of growth can be estimated by means of equation 5

$$K = \frac{FN}{S}.$$

The yeast crop and the amount of fermentation are measured after a convenient amount of fermentation has taken place, the initial seeding of yeast being small. F is determined by a separate experiment. The method is not of much practical value as both N and F have to be determined whilst t the time, a factor most easily measured, is eliminated.

With the culture of yeast growing in wort at 20° it was found that 10^6 yeast cells per c.c. caused a fermentation of 0·620 cm. per hour. In another experiment with growing yeast cells it was found that when the yeast crop was $5{\cdot}2 \times 10^6$ cells per c.c. the fermentation was 14·4 cm. That is

$$F = 0{\cdot}620 \times 10^{-6}, \quad N = 5{\cdot}2 \times 10^6, \quad S = 14{\cdot}4.$$

It follows therefore that

$$0{\cdot}434\,K = 0{\cdot}434\,FN/S = 0{\cdot}097$$

a result in agreement with the previous experiments.

Method 4.

The fourth method involves measurements of the rate of fermentation but has an advantage over method 2, as it allows measurements to be taken in any part of the reaction even where the yeast growth is retarded and nevertheless gives true values of K the constant of unrestricted growth.

The method is as follows: two experiments are made, the medium in each case being seeded with small seedings in a known ratio. If the seedings are small enough the time-fermentation curves are identical except that the one reaction is a definite time behind the other. This time-difference is the time for the one seeding to grow to the other, and from the two values K is easily determined.

If R is the ratio of the two seedings and t_1 and t_2 the times at which the two fermentations reach some definite stage in the reaction, then

$$0{\cdot}434\,K = \frac{1}{t_2 - t_1} \log R.$$

This method was tested in the following manner. Two flasks of wort were seeded with actively growing yeast, one 319 times the amount of the other. 75 c.c. of the wort with the greater seeding were placed in the tube of the apparatus and the manometer connected. Appreciable fermentation

started in 15 hours and several readings were taken at definite times. The first solution was then replaced by the second which in the meantime had been resting in the thermostat. On the next day the times were taken when the manometer showed the same changes in pressure as in the previous experiment. The various differences of time were in good agreement and the value of $0{\cdot}434\,K$ calculated from the average came to 0·106.

TABLE III.

t_1 = time at which the first reaction gives the reading M on the manometer scale.
t_2 = time on the next day when the second reaction reaches the same point.
Temp. = 20°. $R = 319$.

M	t_1	t_2	$t_2 - t_1$
9·4	9·20 a.m.	9·0 a.m.	23 hrs. 40 mins.
10·6	9·50	9·32	23 ,, 42 ,,
11·55	10·10	9·52	23 ,, 42 ,,
12·6	10·35	10·18	23 ,, 43 ,,
13·6	10·55	10·38	23 ,, 43 ,,
		Average	23 hrs. 42 mins. = 23·7 hrs.

$$0{\cdot}434\,K = \frac{\log 319}{23{\cdot}7} = 0{\cdot}106. \quad T = 2{\cdot}85 \text{ hrs.}$$

A comparison of the values of K determined by the different methods shows good agreement.

TABLE IV.

Method	1	gives	$0{\cdot}434\,K = 0{\cdot}102$
,,	2	,,	$0{\cdot}434\,K = 0{\cdot}101$
,,	3	,,	$0{\cdot}434\,K = 0{\cdot}097$
,,	4	,,	$0{\cdot}434\,K = 0{\cdot}106$

It is evident that the rate of growth of the yeast during this period is regular and can be measured accurately by yeast counting or by fermentation. It is of interest to note that the yeast crop at any time is composed mainly of the last few generations of yeast and any dying off of the old yeast cells would hardly affect the value of K. Further if the yeast crop were composed of cells of different activity correct values of K would be obtained by method 2 if the average value of the fermentative power remains constant during the time of measurement, for equation $6a$ involves only ratios of S and s.

The equations and experiments cover only about 2 per cent. of the reaction corresponding to a yeast crop of 10 million cells per c.c., which is about 8 per cent. of the final crop. Growth after this time is measurably retarded, probably mainly by the carbon dioxide which is known to have a considerable retarding influence on yeast growth.

An accurate knowledge of the yeast crop and the fermentation during this early period is however given by these experiments and may be put in the following form.

TABLE V.

Temp. = 20°.
$F = 1{\cdot}2 \times 10^{-14}$ grms. per sec. $= 4{\cdot}3 \times 10^{-11}$ grms. per hour. $0{\cdot}434\,K = 0{\cdot}100$.

Hrs.	Cells per c.c.	G. per c.c. fermented
—	1,000	$1{\cdot}8 \times 10^{-7}$
10	10,000	$1{\cdot}8 \times 10^{-6}$
20	100,000	$1{\cdot}8 \times 10^{-5}$
30	1,000,000	$1{\cdot}8 \times 10^{-4}$
40	10,000,000	$1{\cdot}8 \times 10^{-3}$

These equations and methods of estimating the constant of growth can be applied to other micro-organisms. Methods of counting may not always be suitable, but method 4 is probably of general application. The rate of growth of bacteria which produce acid, for instance, could be determined by following the reaction by titration or by electrical conductivity and methods for other growths could be devised without much difficulty.

One of the principal uses of K is to determine the time between infection and the beginning of the chemical action brought about by the organism. The factors which influence K are the factors which determine this time and in extreme cases say whether a liquid is susceptible to the growth of the micro-organism or not.

The value of K may not remain constant over the period of growth in which one is specially interested but a row of constants is not the aim of the investigation and a consistent though variable K may lead to more important results than a constant one.

Micro-organisms grow not only in liquids but also on solids and in the liquid film covering solids and it is of importance to know whether these equations and methods are applicable to determine rates of growth in such cases.

Some information on the subject was obtained by measuring the rate of growth of this culture of yeast in solid wort-gelatin (10 g. gelatin per 100 c.c. wort). The sterile solid medium was melted and seeded with about 100,000 yeast cells per c.c. 75 c.c. were poured into a bottle which was filled with pieces of glass tubing, the whole being previously sterilised. There still remained about 80 c.c. air space. The melted gelatin was cooled by rotating the bottle under a jet of cold water so that the gelatin as it solidified became distributed over the tubing.

The bottle was then connected to the manometer and the apparatus

exhausted. Measurements of the rate of fermentation were then taken and the constant of growth calculated as in Table II

TABLE VI.

Temp. = 15°	t	$S+s$	$0{\cdot}434\,K$	
	−20	(0·1)	—	
	0	2·05 (S)	—	
	4	3·8	0·067	
	5	4·4	0·066	
	6	5·15	0·067	
	9·1	8·25	0·066	
	9·4	8·65	0·067	
	10·6	10·2	0·065	
	10·8	10·4	0·065	
		Average	0·066	$T = 4{\cdot}6$ hrs.

The rate of growth of a yeast colony developing in wort-gelatin from a single cell therefore follows the logarithmic law. The experiments show that retarding influences do not come into play up to the time the colony consists of 200 yeast cells and probably the colony would grow regularly to a much larger size. It is of interest to note that diffusion would play no controlling part in determining the rate of fermentation until the colony consists of several million yeast cells. Slator and Sand [1910] have made calculations of the size of a yeast cell which would just ferment entirely the whole of the sugar diffusing to it in a stationary liquid. At 30° the radius is calculated to be 8×10^{-2} cm. The volume of such a yeast cell is 8 million times as great as one of radius 4×10^{-4} cm. Diffusion under these conditions would not be a limiting factor in the fermentation by a yeast colony until the colony consists of 8 million cells.

The rate of growth of this culture of yeast in wort gelatin is appreciably higher than in wort itself, the rates being 0·066 and 0·050 respectively.

The investigation shows the possibility of measuring rates of growth when the organism is growing on a solid medium.

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XIX. THE IDENTITY OF TRIMETHYLHISTIDINE (HISTIDINE-BETAINE) FROM VARIOUS SOURCES.

By GEORGE BARGER and ARTHUR JAMES EWINS.

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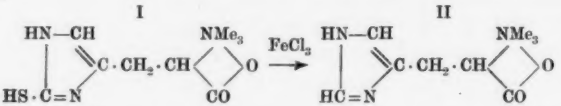
(*Received January 25th, 1913.*)

In a recent paper we showed [Barger and Ewins, 1911] that ergothioneine, a crystalline base containing sulphur, which was isolated from ergot by Tanret [1909], almost certainly possessed the constitution denoted by the formula 1:



Ergothioneine was thus trimethylhistidine (histidine-betaine) containing a sulphur atom attached to a carbon atom of the glyoxaline ring. Further we showed that on oxidation with ferric chloride the sulphur atom was removed as in similar thiolglyoxaline derivatives [Pyman, 1911] and a new base trimethylhistidine or histidine-betaine (II) was produced. We described certain of its salts and pointed out the possibility of its natural occurrence. Kutscher [1910] had previously obtained a crystalline aurichloride of a base from commercial extract of mushrooms, and had stated that this base was possibly trimethylhistidine, since it possessed the formula $C_9H_{15}O_2N_3$ and gave a strong reaction with sodium *p*-diazobenzene sulphonate. As, however, no details were published regarding this salt, we were unable to determine whether our base was identical with Kutscher's.

A short time after our paper there appeared a publication by Reuter [1912] in which he described the isolation of histidine-betaine from *Boletus edulis*, and gave a description of certain of its salts. Among others he characterised two picrates; one, a monopicrate which was analysed, but to which no melting point was assigned, and another, melting at 206° which was not analysed, but which, from a picric acid determination, appeared to

be a dipicrate. The picrate of histidine-betaine obtained by us from ergothioniene melted at 123° and analysed well for the dipicrate. This difference was pointed out by Reuter and for some time we were at a loss for a satisfactory explanation. We now find that the apparent discrepancy was due to the fact that our melting point was determined on the air-dried salt, which contained two molecules of water of crystallisation: our analysis on the other hand was made with an anhydrous specimen of which the melting point was not at the time determined. Quite recently Kutscher [1912] succeeded in synthesising the betaine in question from α-chloro-glyoxaline-propionic acid and trimethylamine at 80°, and by means of the aurichloride established the identity of the synthetic base with the supposed trimethylhistidine previously isolated by him from mushrooms. On repeating Kutscher's synthesis we were able to isolate from the reaction product a small quantity of the picrate of the betaine which melted at 123° as we had found in the case of the base derived from ergothioneine. The two picrates were, indeed, in all respects identical.

At this juncture we communicated with Dr C. Reuter who very kindly supplied us with specimens of the two picrates prepared by him, together with a full description of these salts, for which we offer him our best thanks. We then found that the (anhydrous) dipicrate melting at 205°–206° described by Reuter, crystallised from water with two molecules of water of crystallisation, and, when air dry, melted at 123°–124°, and was then in all respects identical with the picrate obtained by us. The water of crystallisation could be removed by drying *in vacuo* over sulphuric acid, but only with some difficulty. The picrate thus obtained always showed signs of sintering at about 125° (doubtless owing to traces of water still adhering) but did not melt until 205°–206° as described by Reuter. The crystalline anhydrous salt could be readily obtained by recrystallising the picrate (dried *in vacuo*) from absolute alcohol, and then melted at 213°–214°. In a private communication Dr Reuter informed us that a purified specimen of his dipicrate melted at 212°–213°. We carried out determinations of the water of crystallisation present in our picrate (A) and that obtained by recrystallising Reuter's anhydrous dipicrate from water (B) with the following results.

A. 0·2061 (air dry) lost (*in vacuo* over H_2SO_4) 0·0106 $H_2O = 5·14$.
B. 0·1603 ,, ,, ,, ,, 0·0083 $H_2O = 5·17$.

$C_9H_{15}O_2N_3 \cdot (C_6H_3O_7N_3)_2 \cdot 2H_2O$ requires $H_2O = 5·21$ per cent.

The complete identity of the two bases was established by the melting points of the two forms of the dipicrate and of the monopicrate (m.p. 201°–202°) which we prepared from our dipicrate according to Reuter's direction

(treating the aqueous solution of the dipicrate with one molecular proportion of sodium hydrate). The melting points of mixtures in all cases showed no depression.

There can be no doubt, therefore, that the trimethylhistidine (histidine-betaine) obtained by us from ergothioneine is identical with that obtained by Reuter from *Boletus edulis* and with the synthetic base obtained by Kutscher. This result affords a further confirmation of the constitution assigned by us, on other grounds, to ergothioneine.

For the isolation of the betaine of histidine we find that the preparation of the dipicrate is the most convenient method; this salt dissolves in about 25 parts of boiling water and readily crystallises in thin elongated rectangular plates with two molecules of water of crystallisation, and when air dry, melts at 123°–124°.

In our previous paper [1911] the melting point of the aurichloride of the betaine was given as 171°. As the quantity of material at our disposal was at that time very small (a few centigrams only) we were unable to analyse this salt, but now, with a further supply of material, we find that the gold salt when pure melts at 184°, in agreement with the melting point as given by Kutscher and by Reuter.

We also determined the rotation of the base recovered from Reuter's dipicrate with the following result.

Concentration of base (in aqueous solution as hydrochloride) = 0·39 per cent.

Actual rotation measured $\alpha_D = +0{\cdot}40°$ (mean of 6 readings) in 2·2 d.m. tube. Whence $[\alpha]_D = +46{\cdot}5°$.

In conclusion we may point out that the trimethylhistidines obtained from various sources are thus shown to be identical. This substance must be classed with the other naturally occurring betaines from amino-acids, such as those from glycine, proline, oxy-proline, and tryptophane (i.e. ordinary betaine, stachydrine, betonicine and hypaphorine respectively).

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XX. A NOTE ON THE METABOLISM OF NITROGENOUS SUGAR DERIVATIVES.

By JAMES ARTHUR HEWITT.

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(*Received February 11th, 1913.*)

It is very generally accepted that carbohydrates are of fundamental importance in the metabolism of nitrogenous foodstuffs. Loewi [1902], using protein decomposition products which no longer gave the biuret reaction, showed carbohydrate to be necessary for the maintenance of a nitrogen equilibrium. Lesser [1904] subsequently demonstrated the inability of fats to replace the carbohydrate. It has been observed further that glycosuria following excision of the pancreas in dogs is attended by increased excretion of nitrogen [Falta, Grote and Staehelin, 1910], and Taylor and Cathcart [1910] found phloridzin diabetes to be followed by disturbances of nitrogen metabolism. Finally, Falta and Gigon [1908] have concluded that carbohydrate is indispensable for the utilisation of protein, and it has been assumed that some compound is formed between the carbohydrate and the nitrogenous material [Lüthje, 1906].

In an attempt to determine the nature of this synthesis Spiro [1907] showed that the injection of glycine and fructose together gave rise to the appearance in the urine of a dicarboxylic acid normally absent. On the other hand much *in vitro* work has been done and various definite compounds of sugars and nitrogenous substances have been obtained. Lobry de Bruyn [1904] prepared compounds of sugar and ammonia, Schoorl [1900] a urea derivative of glucose, and Morrell and Bellars [1907] have synthesised a product from glucose and guanidine. It may be noted however that in some cases at least the reaction does not seem to be one of simple condensation [Irvine, 1909]. Little seems to have been done on the metabolism of substances of this nature.

Fabian [1899] found that glucosamine is with difficulty oxidised in the body and is in great part excreted unchanged. The extreme stability of this

compound and its great resistance to *in vivo* oxidation are somewhat unexpected; but in light of recent evidence brought forward by Irvine and Hynd [1912] for considering glucosamine to be not a simple derivative of glucose, but a ring compound containing a betaine grouping, Fabian's results are largely explained.

For the purposes of studying the metabolism of compounds such as those mentioned above, those referred to offer certain disadvantages; Lobry de Bruyn's products are somewhat indefinite, the urea derivative difficult to obtain in quantity, and most other compounds of this nature are highly unstable.

For the following experiments, glucose *p*-phenetidide, the condensation product of glucose and *p*-phenetidine, was selected on account of the ease of its preparation and its comparative stability to hydrolytic agents. At the boiling point, however, in solution it is readily hydrolysed by 0·2 % hydrochloric acid.

The preparation of glucose *p*-phenetidide was carried out according to the method devised by Irvine and Gilmour [1909].

The toxicity of the compound is also disputed [St Mostowski, 1909] (quoted from the above-mentioned authors).

White rats were used, the faeces and urine being collected separately in cages similar to those described by Schäfer [1912]. To prevent loss of nitrogen from the urine in the form of ammonia, known volumes of concentrated sulphuric acid were generally added to the receiving vessel previous to collection. The glucose *p*-phenetidide was administered either in the food (bread and milk *ad libitum*), or less often by intra-peritoneal injection. Quantities of from 0·1 to 1 g. (usually 0·5 g.) were given daily. The intake and output of nitrogen were determined both before and during the experiment, but no alteration in the nitrogen equilibrium was observed. The presence of glucose *p*-phenetidide in the food caused no variation in the amount consumed *per diem* which could not be accounted for by the normal difference from day to day.

The urine of those animals receiving the compound was without exception of a much darker colour than that of the controls or of themselves before or after the feeding. Discontinuance of feeding at once restored the normal light yellow colour. The colour is not caused by hydrolysis taking place in the urine by means of the acid present, for when the urine was collected alone the colouration persisted[1].

[1] Oxidation of glucose *p*-phenetidide by means of hydrogen peroxide in neutral solution produces a brownish black syrup of unknown composition.

Injection of 0·1 g. produced similar results, and moreover in each case a substance displaying active reducing properties was excreted in the urine. Greater reduction of Fehling's solution, Barfoed's solution and Nylander's reagent occurred after previous boiling of the urine with mineral acid. In one case following injection, a compound was obtained from the urine by means of phenylhydrazine, which in microscopic appearance resembled the phenylosazone of glucose, but owing to the small quantity confirmation could not be obtained.

It was mentioned above that the toxicity of glucose *p*-phenetidide was disputed, St Mostowski denying the general conclusion that it is poisonous (we are unaware however of the quantities administered by this author).

In these experiments it is shown that in quantities up to 1 g. *per diem* it produces no ill effects in rats of from 220 to 250 g. body weight. Experiments with a view to determining the effects of larger doses were not performed owing to scarcity of material.

Injection of *p*-phenetidine however is followed by marked results. The *p*-phenetidine cannot be given by feeding owing to the animals refusing to eat all food containing it.

In a cat 4 c.c. of an 8 % solution caused a large and rapid fall of blood pressure which returned to normal within a minute and a half and remained steady and regular until death ensued ten minutes later. Post-mortem examination revealed extensive intra-vascular clotting.

It seems probable that much of the glucose *p*-phenetidide escapes hydrolysis or is rendered innocuous by conversion into some other substance, from the facts that in doses of 4 c.c. of an 8 % solution or 0·32 g. per 2·5 kilo body weight, *p*-phenetidine is highly toxic, and that glucose *p*-phenetidide is harmless when given in amounts of 1 g. per 220 g. or 4·5 g. per kilo body weight, equivalent, if complete hydrolysis took place, to about 2 g. *p*-phenetidine.

Summary.

1. After administration of glucose *p*-phenetidide by mouth or by injection, a reducing substance is excreted by the urine.

2. No effects on nitrogen metabolism are produced.

3. In amounts up to 1 g. per 220 to 250 g. body weight *per diem* glucose *p*-phenetidide is not toxic.

4. Some of the glucose *p*-phenetidide escapes oxidation in the body or is converted into a substance of non-poisonous nature.

5. In amounts of 0·32 g. per 2·5 kilo body weight (or 0·108 g. per kilo) *p*-phenetidine is highly toxic.

The author would like to express his indebtedness to Dr Cathcart of Glasgow University for suggesting the line of research, and also to Professor Irvine and to Professor Herring of St Andrews University for kindly advice and criticism.

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XXI. AN ATTEMPT TO ESTIMATE THE VITAMINE-FRACTION IN MILK.

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(*Received February 17th, 1913.*)

The isolation of the beri-beri vitamine from milk [Funk, 1912, 1], together with the important fact observed by Andrews [1912], that infantile beri-beri occurs when the children are fed by mothers suffering from beri-beri, suggest a new line of investigation into the etiology of infantile scurvy and rickets. The problems which present themselves in connection with these investigations have been already set forth [Funk, 1912, 2] and will be fully treated in an account in the *Ergebnisse der Physiologie*, Vol. 13. These problems are briefly the following. (1) What is the normal amount of vitamines in milk of different species, including the human? (2) Is there a definite relationship between the amount of vitamines secreted in the milk and that ingested in the food? This point is of special interest because of the periodical appearance of rickets in winter and disappearance in summer, a period when the cows return to pasture. (3) What effect have boiling and pasteurisation on the vitamine content of milk? All these points await their solution until a method for determining such small quantities is available. The present inquiry shows that the ordinary chemical methods for estimating vitamines can hardly suffice and attention at present must therefore be directed to colorimetric methods.

Nevertheless the results described below give an idea of the amount of vitamines possibly present in milk. The whole vitamine fraction (nitrogen precipitated by phosphotungstic acid in the alcoholic extract of the dried milk) from 1 litre of milk amounts to 1–2·5 mgr. nitrogen. This would correspond to 1–3 cg. vitamine ($C_{17}H_{20}O_7N_2$). After the elimination of the vitamine-fraction the residual nitrogen amounts to 20–50 mgr. per litre milk. This residual nitrogen represents in all probability allantoin; assuming this to be correct, one litre of milk contains 0·06–0·15 grm. allantoin, a figure in good agreement with that obtained by Ackroyd [1911] by means of a direct method, namely 0·199 grm.

The results further indicate that for a chemical investigation of the vitamines of milk it will be necessary to start on a large amount of milk. At the same time they show that the artificial protein-free milk, such as was used by Osborne and Mendel [1912] in their experiments on growth, differs from true protein-free milk in lacking these nitrogenous substances. It is therefore quite conceivable, as the experiments of Hopkins and Neville [1913] point out, that these substances play an important part in the process of growth.

As the figures below show, the milk after removal of fat by centrifuging has lost about 50 % of vitamine and allantoin.

The milks used in these experiments were from London dairies. For each experiment a litre was evaporated in vacuo at 30°, using a distilling flask constructed of two parts from which therefore the residue could be quantitatively removed. The process of drying the residue was hastened in the last stages by an addition of alcohol. The residue was powdered in a mortar and dried in a vacuum desiccator to constant weight. The fine powder was then shaken with a definite amount of alcohol (400 c.c.) in a shaking machine for two hours. The extract was filtered and of the filtrate an aliquot part was evaporated in vacuo. The residue was extracted with water and the extract transferred to a measuring flask. The watery extract was filtered and the filtrate found in every case to be entirely free from proteins. A measured quantity was acidified slightly with sulphuric acid and precipitated with 10 % phosphotungstic acid. After standing for 48 hours the precipitate was filtered and washed with dilute sulphuric acid. In accordance with my previous results this phosphotungstic acid precipitate can be regarded as the vitamine-fraction. Both in the precipitate and filtrate the nitrogen was estimated by Kjeldahl, N/20 normal solutions being used for titration.

		Weight of residue	Nitrogen of the vitamine fraction in mgr. (phosphotungstic acid precipitate)	Residual nitrogen in mgr. (phosphotungstic acid filtrate)
1.	Centrifuged milk	100	2·54	50·9
	Control	99·5	2·46	49·6
2.	Uncentrif.	133	1·7	30·7
	Centrif.	98	1·53	16·8
3.	Uncentrif.	127	2·5	22·7
	Centrif.	90	1·4	12·1
4.	Uncentrif.	129	1·96	29·6
	Centrif.	92	1·2	15·3
5.	Uncentrif.	132	2·4	49·2
	Centrif.	96	1·6	27·3
6.	Centrif.	89	1·06	22·4
7.	Centrif.	105	1·83	44·9

In this connection the behaviour of allantoin when boiled with Ruhemann's reagent (triketohydrindene hydrate) should be noted. According to Abderhalden and Schmidt [1911] allantoin when boiled for one minute with Ruhemann's reagent does not give any colouration. But although this is so, if the boiling be continued for 2–3 minutes a violet colouration appears and this reaction might be used as a test for allantoin. Since the crude product obtained by oxidation of uric acid by permanganate gave the reaction at once, probably due to admixture with allanturic acid, and allantoin is known to be destroyed by boiling, it was thought that a decomposition product of allantoin, probably allanturic acid, was the cause of this reaction. A sample of pure allantoin was therefore boiled with PbO_2 and another with water. Both samples gave a strong allanturic acid reaction after a certain time of boiling, the sample with PbO_2 giving however the stronger reaction. The reaction seemed to disappear after prolonged boiling. The reaction may also be obtained from hydantoin.

It is interesting to note, that we have in allantoin an example of a substance, which like vitamine is destroyed by boiling.

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XXII. THE ENZYMES OF WASHED ZYMIN AND DRIED YEAST (LEBEDEW). I. CARBOXYLASE.

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(*Received February 17th, 1913.*)

When zymin is thoroughly washed with water a residue is left, which no longer has the power of fermenting glucose, but regains this power when the washings are added to it, even when the latter have been boiled [Harden and Young, 1910]. The dried yeast used by Lebedew behaves in a precisely similar manner [Euler and Bäckström, 1912].

It is therefore a matter of considerable interest to ascertain how far this treatment affects the various enzymes which are known to exist in zymin and dried yeast. It can thus be ascertained whether these enzymes are themselves soluble and whether if insoluble they require for their action the presence of a soluble substance of the nature of a coenzyme.

It is also probable that some light may be thrown on the possible function of some of these enzymes in the process of alcoholic fermentation.

Carboxylase.

Since the discovery by Neuberg and Hildesheimer [1911] of the unexpected fact that yeast, yeast juice and zymin readily and rapidly decompose pyruvic acid and other α-ketonic acids with evolution of carbon dioxide and formation of an aldehyde, the opinion has been expressed in many quarters that pyruvic acid may form a stage in the enzymatic decomposition of glucose into alcohol and carbon dioxide [Neubauer and Fromherz, 1911; Neuberg and Kerb, 1912; Kostytschew, 1912; Lebedew 1912].

On the other hand it is possible that carboxylase is quite independent of the enzymes of alcoholic fermentation, its function being that of decomposing the α-ketonic acids formed by the deaminisation of the α-amino-acids (see Neubauer and Fromherz, 1911).

In order to examine the action of washed yeast preparations on pyruvic acid, experiments were made not only on the free acid but also on the

sodium salt in presence of weak inorganic acids, by the aid of which the acidity was diminished, whilst at the same time the whole or almost the whole of the carbon dioxide produced was evolved.

The evolution of carbon dioxide was observed by the aid of the apparatus previously described [Harden, Thompson and Young, 1910]. No quantitative estimations of acetaldehyde were made, but its presence was proved by the reaction with Schiff's reagent in all cases in which an evolution of carbon dioxide was observed. Control experiments were at the same time carried out with glucose and phosphate. It was thus found that the residue obtained by washing zymin and dried yeast (Lebedew) until they could no longer ferment glucose, was capable of decomposing pyruvic acid quite readily. The interesting fact is thus ascertained that carboxylase does not require the presence of a coenzyme removable by washing in order to exert its characteristic reaction on pyruvic acid. This result does not however allow any definite conclusion to be drawn as to the possible function of carboxylase in alcoholic fermentation. It can only be concluded that if the decomposition of pyruvic acid actually be a stage in the alcoholic fermentation of glucose, the soluble coenzyme is required for some change precedent to this, so that in its absence the production of pyruvic acid cannot be effected. The following experiments illustrate the results obtained. The solutions throughout were saturated with carbon dioxide at the temperature of the bath before the commencement of incubation.

Exp. 1. A sample of Schroder's dried yeast (nach Lebedew) was washed 3 times on the centrifuge with water and then brought into acetone and dried. The following experiments were then made.

(*a*) 2 g. washed and acetoned yeast + 10 c.c. 1 per cent. pyruvic acid + 40 c.c. H_2O.

(*b*) 2 g. washed and acetoned yeast + 25 c.c. 1 per cent. pyruvic acid + 25 c.c. H_2O.

(*c*) 2 g. washed and acetoned yeast + 25 c.c. 1 per cent. pyruvic acid + 5 c.c. 0·3 molar Na_2HPO_4 + 20 c.c. H_2O.

(*d*) 2 g. washed and acetoned yeast + 0 pyruvic acid + 5 c.c. 0·3 molar Na_2HPO_4 + 45 c.c. H_2O + 2 g. glucose.

These were incubated at 25° and the evolution of CO_2 measured.

Time	(*a*)	(*b*)	(*c*)	(*d*)
1 hr. 50 mins.	3·5	1·6	18·6	1·9

The action of the washed yeast on glucose was extremely small (*d*) and very little fermentation of the free pyruvic acid (*a* and *b*) occurred. In presence of sodium phosphate however a considerable evolution of CO_2

occurred (18·6 c.c.) and a strong reaction for acetaldehyde was given by the filtrate. The small effect on free pyruvic acid is ascribed to the effect of the acidity of the solution on the enzyme; in presence of sodium phosphate, on the other hand, the acidity is mainly due to NaH_2PO_4 formed by interaction of the pyruvic acid with the Na_2HPO_4.

Exp. 2. A similar experiment was made with similar results using the same washed and acetoned yeast, but washing it again 3 times to remove the last trace of coenzyme.

10 g. of acetoned yeast were washed and made up to 85 c.c.

(*a*) 25 c.c. yeast suspension + 21 c.c. 1 per cent. pyruvic acid + 7 c.c. H_2O.

(*b*) 25 c.c. yeast suspension + 21 c.c. 1 per cent. pyruvic acid + 7 c.c. 0·3 molar Na_2HPO_4 (approx. equivalent to the pyruvic acid).

(*c*) 25 c.c. yeast suspension + 21 c.c. H_2O + 7 c.c. 0·3 molar Na_2HPO_4 + 2 g. glucose.

Time	(*a*)	(*b*)	(*c*)
55 mins.	4·1	22·0	1·2 c.c.

Here again the action on the free acid is small, whereas a considerable evolution of CO_2 occurs in presence of phosphate. The amount produced is really greater than that evolved since when pyruvic acid and sodium phosphate are present in equivalent amount a portion of the CO_2 is retained as $NaHCO_3$ in equilibrium with NaH_2PO_4.

$$CH_3 \cdot CO \cdot COOH + Na_2HPO_4 \rightarrow CH_3 \cdot CO \cdot COONa + NaH_2PO_4 \rightarrow$$
$$CH_3 \cdot CHO + NaHCO_3 + NaH_2PO_4 \rightleftarrows CH_3 \cdot CHO + Na_2HPO_4 + CO_2 + H_2O.$$

Accordingly, the combined CO_2 was determined after incubation and, in a separate sample, before incubation and it was thus found that 12·9 c.c. of CO_2 were retained and should be added to the amount observed in (*b*), making a total production of 34·9 c.c.

Exp. 3. The effects of neutral potassium citrate and of excess of free boric acid in presence of the sodium salt of pyruvic acid were tried. The yeast employed was a similar preparation to that used in Exp. 2.

(*a*) 25 c.c. yeast suspension + 21 c.c. 1 per cent. pyruvic acid + 7 c.c. 0·3 M. potassium citrate neutral to phenolphthalein.

(*b*) 25 c.c. yeast suspension + 21 c.c. H_2O + 7 c.c. 0·3 M. sodium phosphate + 2 g. glucose.

(*c*) 25 c.c. yeast suspension + 21 c.c. H_2O + 7 c.c. 0·3 M.K. citrate.

(*d*) „ „ + 21 c.c. 1 per cent. pyruvic acid + 2·4 c.c. N. KHO + 3 g. H_3BO_3.

Time	(*a*)	(*b*)	(*c*)	(*d*)
1 hr. 25 mins.	4·5	1·3	1·0	25·6

The sample of yeast had practically no action on glucose (*b*), comparatively little on pyruvic acid in presence of citrate (*a*) and a large action in presence of free boric acid (*d*). This result therefore is in agreement with the idea that the action of the enzyme is greatly inhibited by acid, citric acid being much stronger than boric acid.

Exp. 4. 10 g. dried yeast was washed 3 times and made to 100 c.c.

(*a*) 25 c.c. yeast suspension + 25 c.c. 1 per cent. pyruvic acid + 2·5 c.c. N. KHO + 3 g. boric acid + toluene.

(*b*) 25 c.c. yeast suspension + 21 c.c. H_2O + 5 c.c. 0·3 M. Na_2HPO_4 + 2 g. glucose + toluene.

Time	(*a*)	(*b*)
1 hour	21·04	0·2 c.c.

Here again a good fermentation of pyruvic acid is produced by a sample of washed yeast incapable of fermenting glucose, the total evolution from which in 18 hours was only 0·4 c.c.

Exp. 5. 20 g. zymin (Schroder) were washed 3 times and made to 100 c.c.

(*a*) 25 c.c. yeast suspension + 22·5 c.c. H_2O + 2 g. glucose + 5 c.c. 0·3 M. Na_2HPO_4.

(*b*) „ „ + 25 c.c. 1 per cent. pyruvic acid + 2·5 c.c. N. KHO + 3 g. boric acid.

Time	(*a*)	(*b*)
43 mins.	17·8	0·8
18 hours	22·7	0·8

Washed zymin therefore has the same action as washed dried yeast.

Summary.

Zymin and dried yeast (Lebedew) after being freed from coenzyme by washing and thus rendered incapable of fermenting glucose, readily decompose pyruvic acid into carbon dioxide and acetaldehyde, provided that the acidity of the solution is kept low.

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